EVALUATION OF NEW POSTHARVEST FUNGICIDES FOR THE CONTROL OF PHYTOPHTHORA BROWN ROT

by

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DECLARATION

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SUMMARY

Brown rot is a citrus postharvest disease caused by *Phytophthora* spp. during continuous wet conditions. Fruit closest to the soil surface in the orchard are usually infected when the infecting propagules present are splashed upward during irrigation or rain. When infections on fruit are still in early development, it may go unnoticed when fruit are harvested. Infections develop further after harvest and can infect other fruit while in storage or transit. In the current study, the efficacy of actives azoxystrobin, fludioxonil and potassium phosphite was tested for the control of postharvest Phytophthora brown rot on citrus, as there is currently nothing registered for the management of this disease in South Africa.

The fungicide sensitivities of 121 *Phytophthora nicotianae* isolates belonging to either a previously unexposed population or previously possibly exposed population, were tested for against the strobilurin azoxystrobin, based on the growth of mycelium. The mycelial growth on corn meal agar (CMA) amended with azoxystrobin at 0-, 0.25-, 0.5-, 1-, 10-, 100-, and 2000 µg/ml with the addition of 100 µg/ml salicylhydroxamic acid (SHAM), to inhibit the alternative respiration route, was measured after 7 days. The addition of SHAM did not have a significant effect on the growth of mycelia. CMA was also amended with the phenylpyrrole fludioxonil at 0-, 1-, 100-, 1000-, and 10 000 µg/ml. The effective concentration for 50% reduction of mycelial growth (EC_{50}) for azoxystrobin ranged from 0.01 to 0.46 µg/ml for both populations and the EC_{90} ranged from 4.28 to 84.85 µg/ml for both populations. Fludioxonil sensitivity had a much wider range and higher EC values. The EC_{50} for both populations ranged from 3.10 to 1613.52 µg/ml and the EC_{90} values were 1090.50 to 9929 µg/ml.

Subsequently, curative and protective actions of aqueous postharvest dip treatments, were carried out with the use of azoxystrobin at 1125 µg/ml and fludioxonil at 598 µg/ml. With the *in vivo* trials, potassium phosphite was added as an additional treatment at 1500 µg/ml. These trials were repeated on three fruit types namely lemons, oranges and mandarins and at four time intervals. The data clearly showed that with all three fungicides, the curative efficacy is excellent when treatments occurred up to 12 hrs after inoculation. Azoxystrobin and potassium phosphite exhibited excellent protective activity up to 48 hrs between treatment and inoculation. An overall trend that was seen with all three fruit types, was that the longer the fungicide was present on the fruit, the better the protective activity. The nesting data clearly demonstrated that only azoxystrobin amended wax significantly reduced brown rot from spreading to the healthy fruit, when compared to the control.

With this study, it could be seen that all three fungicides, with their different actives, have the potential to effectively manage postharvest brown rot. Considering that azoxystrobin and fludioxonil are already registered on citrus for the postharvest use of *Penicillium* management,

and potassium phosphite that are registered as a preharvest *Phytophthora* treatment, this study indicate that they will also provide added protection against Phytophthora brown rot.

OPSOMMING

Bruin vrot is ‘n sitrus na-oes siekte wat veroorsaak word deur *Phytophthora* spesies gedurende aanhoudende nat toestande. Die vrugte naaste aan die grond oppervlakte in boorde word gewoonlik geïnfekteer wanneer die infekterende propagules opwaarts gespat word tydens besproeiing of wanneer dit reën. Wanneer infeksie op vrugte nog in vroeë ontwikkeling is, kan dié vrugte ongesiens geoes word en ander vrugte tydens die stoor daarvan, of tydens vervoer, infekteer. Die huidige studie toets die effektiwiteit van aktiewes asoksistrobien, fludioxonil en kaliumfosfiet vir die beheer van na-oes Phytophthora bruin vrot op sitrus, aangesien daar tans niks geregistreer is vir die beheer van hierdie siekte in Suid-Afrika nie.

Die swamdoder sensitiwiteit van 121 *Phytophthora nicotianae* isolate wat behoort aan nie-blootgestelde- en moontlik blootgestelde populasies, was gebaseer op die groei van die miselium. Die miselium groei op mieliemeel agar (MMA) met die byvoeging van die strobilurien asoksistrobien teen 0-, 0.25-, 0.5-, 1-, 10-, 100-, en 2000 µg/ml en addisioneel 100 µg/ml salisielhidroksamiensuur (SHAM), om alternatiewe respirasie roetes the inhibeer, was gemeet na sewe dae. Die byvoeging van SHAM het geen betekenisvolle effek gehad op die miselium se groei nie. KMA was ook bygevoeg met die finielpirool fludioxonil teen 0-, 1-, 100-, 1000-, en 10 000 µg/ml. Die effektiewe konsentrasie om die miselium se groei met 50% te verminder (EC_{50}) was vir asoksiestrobien tussen 0.01 en 0.46 µg/ml vir beide populasies en die EC_{90} was tussen 4.28 en 84.85 µg/ml vir beide populasies. Fludioxonil sensitiwiteit het ‘n veel wyer reeks en hoër EC waardes gehad. Die EC_{50} vir beide populasies was tussen 3.10 en 1613.52 µg/ml en die EC_{90} waardes was tussen 1090.50 en 9929 µg/ml.

Gevolglik was die genesende en beskermde aksies getoets met die gebruik van ‘n na-oes doop en was uitgevoer met asoksiestrobien teen 1125 µg/ml en fludioxonil teen 598 µg/ml. Met die *in vivo* proewe was kalium fosfiet teen 1500 µg/ml bygevoeg. Hierdie proewe was getoets op drie vrug tipes, naamlik suurlemoene, lemoene en mandaryne en teen vier tyd intervalle. Die data wys duidelik dat al drie swamdoders goeie genesende aksies het wanneer behandeling plaasvind tot en met 12 ure na inokulasie. Asoksietrobien en kalium fosfiet het goeie beskermende effektiwiteit gewys tot en met 48 uur tussen behandeling en inokulasie. ‘n Algehele tendens wat opgemerk was met al drie vrug tipes, was dat hoe langer die swamdoder teenwoordig was op die vrug voor inokulasie, hoe beter was die beskermende effek van die swamdoder. Die voorkoming van oordrag data het gedemonstreer dat net asoksiestrobien wat in waks bygevoeg was, het die verspreiding van bruin vrot na gesonde vrugte betekenisvol verminder het, as dit met die kontrole vergelyk was.

Met hierdie studie, kan daar gesien word dat al drie van die swamdoders, met hul verskillende aktiewes, effektief aangewend kan word vir na-oes bruin vrot beheer. Aangesien

asoksiestrobien en fludioxonil klaar in Suid-Afrika geregistreer is vir na-oes *Penicillium* beheer op sitrus, en kalium fosfiet vir voor-oes *Phytophthora* beheer, kan hierdie studie aandui dat die swamdoders addisionele beskerming bied teen na-oes Phytophthora bruin vrot.

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CONTENTS

CHAPTER 1

Phytophthora diseases of citrus and their management

INTRODUCTION

Phytophthora de Bary has been characterised as a plant destroyer, with good reason. Over 150 species have been identified during the 120 years since Anton de Bary described this genus (Kroon *et al*., 2012). The number of identified species has doubled in the last decade alone due to identification technology advancements and even more undescribed species are expected to be identified in the nearby future (Kroon *et al*., 2012; Yang *et al*, 2017). The best known *Phytophthora* species is *P. infestans* which had devastating effects in Ireland in 1845, causing the well-known potato famine (Meng *et al.*, 2014; Yang *et al*., 2017). Even before *P. infestans* caused the potato famine, there were records of large-scale destruction of citrus plants in 1836 on the Azore Islands. The first recorded *Phytophthora* epidemic on citrus occurred during 1832-1836 (Gade and Lad, 2018). *Phytophthora,* which also includes green plants and algae, belongs to the kingdom Straminipila, class Oomycetes, order Peronosporales and the family Peronosporaceae (Meng *et al.,* 2014).

The class Oomycota are not seen as true fungi based on several features and are more phylogenetically related to plants and algae. Cell walls (septa) in the hyphae do not occur frequently, resulting in coenocytic hyphae, which means multiple nuclei. Oomycetes are mostly diploid whereas true fungi are haploid (Meng *et al*., 2014). Oomycota do not have chitin in their cell walls, which are characteristic of true fungi, but are rather composed of mainly β-1,6 and β-1,3 glucans (cellulose). Oomycetes are unable to synthesize β-hydroxysterols (Meng *et al.*, 2014). Several oomycetes produce sporangia, which produce biflagellated swimming spores, termed zoospores that are released when the environmental conditions are favourable.

This genus is universally present with a wide host range and occurs in natural-, horticultural- and agricultural systems (Burgess *et al*., 2017). *Phytophthora* spp. with very broad host ranges includes *P. ramorum*, *P. nicotianae* and *P. cinnamomi,* while other have narrow host ranges such as *P. sojae* and *P. infestans* (Hyde *et al.*, 2014). Kamoun *et al*. (2015) undertook a survey to establish the top 10 most important oomycetes worldwide with reference to the economic impact and scientific importance. *Phytophthora* spp. were found to occupy six positions out of the top 10. *Phytophthora infestans* was ranked nr. 1 and *P. ramorum* as nr. 2, *P. sojae* nr. 4, *P. capsici* nr. 5, *P. cinnamomi* nr. 7 and *P. parasitica* (synonomous to *P. nicotianae*) as nr. 8.

To identify *Phytophthora* spp. in any given region, a good taxonomic system is needed. Conventionally, identification was based on morphological characterization, which, among other, included homothallism, morphology of sporangia, and the configuration of antheridia. With the use of molecular identification and DNA (deoxyribonucleic acid) sequencing, the concept of taxonomic phylogeny shed more light on the relation between species. According to Yang *et al*. (2017), Waterhouse initially formed six groups of *Phytophthora* in 1963 that were only based on morphological characteristics. However, this was not accurate due to many species having overlapping morphological characteristics.

With the analysis of the internal transcribed spacer (ITS) region, the first molecular phylogeny for *Phytophthora* was constructed in 2000 with the use of 51 species (Cooke *et al*., 2000). Blair *et al*. (2008) constructed a more detailed and correct phylogeny that identified 10 clades by using seven genetic markers and 82 species. Martin *et al.* (2014) used 90 species and 17 potential species, to construct a mainly similar phylogeny. Yang *et al.* (2017) constructed a phylogeny with 142 confirmed and described species and 43 provisionally named species, which clearly demonstrated how fast this genus expanded and that, to understand the evolution of a genus, molecular phylogeny is vital.

Species within the genus *Citrus* occur worldwide and considered to be one of the most important fruit crops, which consists of 1300 species in 140 genera in the family Rutaceae (Savita *et al*., 2012). This economically important perennial fruit crop is grown in over 100 countries. The citrus industry is important in South Africa as it yields a total income of R20 billion per year (CGA, 2019), and severe losses to the citrus industry can be caused by soilborne *Phytophthora* pathogens that cause devastating diseases.

Phytophthora can occur in dry areas as well as high rainfall and well irrigated production areas. It is considered an endemic pathogen in most citrus producing regions and are deemed to be universally present as the *Phytophthora* populations persists in the soil if the fibrous roots are repeatedly infected (Graham and Menge, 2000). Diseases such as root rot, gummosis (foot rot), damping-off of seedlings in nurseries, leaf fall, branch cankers, brown rot of fruit and postharvest decay in packing cartons are associated with these pathogens (Graham and Menge, 2000; Graham and Feichtenberger, 2015).

According to Bawage *et al*. (2013) there are 12 *Phytophthora* spp. infecting citrus globally, namely, *P. nicotianae, P. cinnamomi, P. capsici, P. citrophthora, P. citricola, P. boehmeriae, P. palmivora, P. syringae, P. megasperma, P. drechsleri, P. cactorum,* and *P. hibernalis.* These different species thrive in different environments which is why they are so widely distributed (Ahmed *et al*., 2012). The behaviour of each species differ in response to various aspects including polyphagy, fungicide sensitivity and specific temperatures required for optimal mycelial growth, which result in only one species usually dominating in a certain growing area (Yaseen e*t al.,* 2010).

P. palmivora, P. nicotianae and *P. citrophthora* produce papillated sporangia and when the environmental conditions are favourable, zoospores that are mainly biflagellate, are released in abundance. The sporangia produced by the heterothallic *P. nicotianae* are 30-40 x 38-50 µm, usually spheroid, papillate and non-caducous (Das *et al.*, 2016), and the ideal mycelial growth temperature is 30-32°C. In contrast, *P. palmivora* has caducous sporangium which are papillated and can be ovoid, ellipsoid or obpyriform in shape (25-35 x 40-60 µm) (Das *et al.*, 2016), and this pathogen's mycelia has an optimum growth temperature that is between 27-30°C. Characteristically, *P. palmivora* sporangia have short pedicels with a mean of 50 x 28 µm (Tashiro *et al.,* 2012). These two are heterothallic pathogens, with spherical oogonia and amphigynous antheridia (Das *et al.*, 2016). Both can produce non-papillated, oval/spherical and thick walled chlamydospores (terminally or intercalary) and oospores with diameters 24.2-38.8 µm and 22-29 µm, respectively (Das *et al.*, 2016).

Phytophthora nicotianae's oogonia do not fill the oogonia (aplerotic) and *P. palmivora* is almost plerotic (Tashiro *et al.*, 2012). *P. citrophthora,* with optimum mycelial growth temperature of 24-28°C, produce sporangia (27-60 x 45-90 µm) that are usually papillate, noncaducous and are known for being bipapillate. Their shapes vary from spherical, obpyriform, ovoid, obturbinate to ellipsoid (Mounde *et al*., 2012). Some isolates of *P. citrophthora* often do not produce chlamydospores when in culture and do not produce oospores in general. It is important to note that the size and shape of sporangia differ in a species because of the genetic diversity between isolates. A possible source of variation in genetics lies with oospores and, with resting chlamydospores, aids in the survival of the pathogen in unfavourable environmental conditions in infested soil and plant tissue (Kamoun *et al.*, 2015).

Phytophthora citrophthora was the most prevalent species in citrus orchards in areas such as Kenya (Mounde *et al*., 2009), Ghana (Brentu and Vicent, 2015), Spain (Alvarez *et al*., 2008), China (Zheng and Ward, 1998), Australia, Sicily (Yaseen *et al*., 2010), and Syria (Yaseen *et al*., 2010) as well as Indonesia but in combination with *Botryodiplodia theobromae* (teleomorph *Botryosphaeria rhodina*) (Henuk *et al*., 2017). Five *Phytophthora* spp. have been identified in South America occurring on citrus namely *P. parasitica, P. cactorum, P. cinnamomi, P. palmivora* and *P. citrophthora* (Fawcett and Bitancourt, 2003). It is hypothesized that *P. citrophthora* is favoured by a Mediterranean type climate that favours the pathogen in colder months (Yaseen *et al*., 2010).

Phytophthora nicotianae is the species that is dominant in Egyptian citrus nurseries, as it was constantly isolated from diseased trees. However, when the soil had lower temperatures, *P. citrophthora* was more dominant but it gradually decreased when the soil heated up. *P. palmivora* were only occasionally isolated from the nurseries. This confirms that *P. nicotianae* infects citrus roots when they contain sugars during root flushing and that *P. citrophthora*

infects the roots in more cooler temperatures as it can metabolize the starch that are present in the roots at that time. Winter soils are usually too cold for *P. nicotianae* with an optimum temperature requirement of 31°C and summer soils are too hot for *P. citrophthora* with an optimum temperature requirement of 26°C (Ahmed *et al.*, 2012). Therefore it is stated that the species distribution is dependent on the environmental and temporal conditions as *P. citrophthora* frequently occurs in Mediterranean areas and *P. nicotianae* in subtropical areas, while *P. palmivora* is common in tropical and subtropical areas (Tennant *et al*., 2009).

Phytophthora nicotianae was the most prevalent species found in citrus producing areas such as Tunisia (Boughalleb-M'hamdi *et al*., 2017), Kerman province located in Iran (Sadeghy *et al*., 2014), India (Das *et al*., 2016), Thailand and Southeast Asia. The latter is also prevalent with *P. palmivora,* which is the more aggressive pathogen in that area (Hung *et al*., 2015). In South Africa, 162 *Phytophthora* spp. isolates were obtained from seven provinces (Meitz-Hopkins *et al*., 2014). With the use of morphological and molecular analysis, *P. nicotianae* was identified as the dominant species in each province followed by *P. citrophthora* while *P. multivora* were also detected (Meitz-Hopkins *et al*., 2014). *P. nicotianae* is a growing threat to the global trade of nursery plants which can spread resistant *P. nicotianae* isolates to other parts of the world. With global warming becoming more of a problem and as this species thrives in more warmer climates compared to other species, it is possible that this species can expand its geographic distribution (Kamoun *et al.*, 2015).

Thus, the most important *Phytophthora* species that occur on citrus, are *P. nicotianae*, *P. palmivora* and *P. citrophthora* (Savita and Avinash, 2012) and the causal agents of several serious diseases of citrus (Gade and Lad, 2018). It can spread via motile zoospores in water and sporangia that are dispersed through rain, wind and irrigation water splashes. It enters bud joints and wounds and effectively infect trunks, branches, leaves and fruit (Reddy and Murti, 1985). These infections can be controlled with various measures such as cultural practices, biological agents and chemical products, which can be used in combination to achieve the goal in effective farming which is to produce as much healthy crops in a growing season as possible.

PHYTOPHTHORA DISEASES ON CITRUS

Phytophthora crown, root - and foot rot

Introduction

Crown, root and foot rot are usually associated with inadequate irrigation systems and poorly drained orchards as well as during wetter periods (Alvarez *et al*., 2008). If susceptible rootstocks are used, crown rot subsequently occurs when the rootstock is infected below the

soil level (Dewdney, 2019). Root rot of citrus occurs when infecting propagules get access into the root tips due to attraction by root exudates. *Phytophthora* infects a broad host range and includes forest trees and woody plants and when root infection occurs, the infestation usually moves to adjacent trees as this genus produce motile spores that infects and spreads in such a manner (Linde *et al.*, 1994). In South Africa, root rot diseases on woody plants are found on wattle and several eucalyptus and pine species, where *P. cinnamomi* is prevalent (Linde *et al*., 1994). Root rot of citrus trees in South Africa, which leads to the decline of trees, is a problem in all the major citrus producing provinces; namely Limpopo, Mpumalanga and Western- and Eastern Cape (Thompson *et al*., 1993).

Foot rot, which are also known as gummosis, are considered to be the most serious *Phytophthora* disease (Graham and Menge, 2000; Graham and Feichtenberger, 2015). When gummosis occurs on citrus trees it is seen as sap oozing from cankers and wounds on the trunk from infected scions near the soil level (Dewdney, 2019). Furthermore, with the use of tolerant rootstocks, these infected scions can extend downwards to the bud union. An integrated management approach should be followed to control these diseases. This includes proper cultural practices and chemical management although the use of chemicals should be the last management strategy (Dewdney, 2019).

Etiology

Phytophthora nicotianae, P. palmivora, P. syringae, P. citrophthora, and *P. cryptogea* are associated with citrus gummosis (Mounde *et al.*, 2009; Brentu and Vicent, 2015; Henuk *et al.*, 2017; Boughalleb-M'hamdi *et al.*, 2017). In South Africa, feeder root rot is mainly associated with *P. nicotianae* (Thompson *et al*., 1993; Maseko and Coutinho, 2002). The production area determines what species prevail: for instance, in colder production areas *P. citrophthora* is the causal organism of root rot and gummosis but in warmer areas it is *P. nicotianae* (Erwin and Ribeiro, 1996).

Symptoms

Crown, and root rot are similar in some ways but can be distinguished by how the disease manifests itself. Crown rot symptoms are more easily detected than root rot symptoms. When the bark of the tree is infected below the soil level, it is usually due to the susceptibility of the rootstocks. Susceptible rootstock cultivars exude gum and infection usually occurs when the tree is planted too deep or if any other wound occurs on the trunk near the soil level. The rest of the symptoms are similar to foot rot where the bark dries out and cracks which leads to the tree being girdled and the canopy displaying twig dieback, yellowing of leaves and eventual tree death (Le Roux, 2003).

Root rot symptoms usually goes unnoticed in an orchard as the symptoms are not clearly defined. More substantial economic losses of the citrus industry of Southern Africa is caused by feeder root rot in comparison to crown rot. *Phytophthora* infection of root tips usually result in discolouration and the softening of the roots. Infected roots have a water-soaked appearance with the cortex of the root turning soft. This is followed by shedding of the cortex where after only the white root stele remains (Le Roux, 2003; Hung *et al*., 2015). In orchards and nurseries that are heavily infested with *Phytophthora* spp., root rot can be very severe on rootstocks that are susceptible, especially on young trees (Savita and Avinash, 2012). The tree canopy will decline with yellowing of the foliage and in time, twig dieback. Under severe infections of the roots, branch dieback and eventual tree death occur as new roots cannot form quickly enough to replace the old roots that are dying (Le Roux, 2003; Savita and Avinash, 2012). From an economic perspective, Le Roux (2003) and Savita and Avinash (2012) stated that fruit yield is lower and fruit sizes are smaller on infected trees. The smaller fruit are due to less mineral and water uptake because of less roots. This is coupled with depletion of reserves, such as carbohydrates, leading to lower yields (Graham and Menge, 2000).

Foot rot lesions usually develop when infections occur at natural openings in the bark or wounds, relatively close to the soil level. It spreads downward into the roots or up the trunk and onto main and/or secondary branches (Alvarez *et al*., 2008; Savita and Avinash, 2012; Graham and Feichtenberger, 2015). Citrus sap oozes out of small or larger cracks in the outer bark, which remains firm, but the cambium and inner bark are usually damaged. In dry environmental conditions, the gum remains but can disappear after rain as it is water soluble (Savita and Avinash, 2012; Graham and Feichtenberger, 2015). Young trees are more readily infected (Savita and Avinash, 2012) and Graham and Feichtenberger (2015) further states that infections can spread rapidly through young orchard trees as well as nursery trees. The trunks, main- or secondary branches are slowly girdled, and the tree subsequently display pale leaves with yellowish veins. Trees are girdled and killed in incidences of severe infection (Boughalleb-M'hamdi *et al*., 2017). Lesions form on the scions of resistant rootstocks, however with the susceptible rootstocks, they can be found on both the scion and the rootstock (Graham and Menge, 2000). If the lesions do not expand, the oomycete will eventually die, and the affected area will be surrounded by callus tissue.

Phytophthora branch canker

Introduction

Cankers are areas of various shapes or sizes on woody plants that are blistered or dead. These are mainly formed when fungi or bacteria enter a wound or natural opening (Burgess

et al., 2016). Cankers mainly occur on woody trees such as apple, citrus, pear, peach and forest trees. Cankers can cause dieback of branches and twigs and lower fruit yield. In severe cases the trees may die if girdling of the trunk occurs. Phytophthora branch canker and dieback reduce the productiveness of an orchard only a few years after the initial infection is detected (Alvarez *et al*., 2009). Schutte and Botha (2010) suggested that this disease should rather be referred to as trunk *and* branch canker and not only branch canker. *Citrus psorosis virus* (CPsV) can be asymptomatic in citrus trees but when symptoms occurs, it is very similar to *Phytophthora* bark infections (Achachi *et al*., 2014) and can be easily misdiagnosed as a *Phytophthora* bark infection.

It was found that clementine orchards are significantly more associated with Phytophthora branch canker (Alvarez *et al.*, 2008; Schutte and Botha, 2010; Alvarez *et al*., 2011; Vicent *et al*., 2012). Thus, clementine mandarin is regarded as the most susceptible to this disease. Branch canker of citrus trees is a concern for citrus producers, as in cases of inadequate management strategies which can lead to severe tree losses (Alvarez *et al*., 2008).

Etiology

Phytophthora species causing branch canker on citrus include *P. citrophthora* (Alvarez *et al.*, 2008; Schutte and Botha, 2010; Vicent *et al.*, 2012), *P. nicotianae* and *P. citricola* (Alvarez *et al.*, 2008) but *P. citrophthora* is the dominating pathogen in the various studies of branch canker on citrus.

Symptoms

External canker symptoms can be hardly noticeable or clearly visible on most citrus cultivars. At the infected area, lesions develop which are dark with a water-soaked appearance. These lesions can exudate gum that are pale and yellowish. If the disease is advanced, the bark adjacent to the infected area becomes soft and has a yellow colour. Infected bark dries and splits, with dead bark tending to fall off and exposing brown necrotic stains under the bark (Graham and Menge, 2000). Decaying wood under the infected bark can have a distinctive odour. In cases where advanced disease development has occurred, the infected area can stretch around the tree trunk and girdle it over time. This results in trees starting to wilt with foliage and fruit yield reduced significantly as it drops to the ground (Mariau, 2001).

Cankers appear as v-shaped stains on the trunks and extends downwardly to the bud union and upwards to the main branches and secondary branches, with a clear distinction between healthy and infected tissue (Alvarez *et al*., 2008; Schutte and Botha, 2010). Rootstocks of infected trees usually remain healthy in contrast to the cankers on the scions. These can kill the infected branches and in extreme disease cases, the entire tree, which leads to shedding of the rest of the leaves and fruit (Vicent *et al*., 2012). The rate at which

cankers develop depends on the physiological state of the tree as well as the climate (Mariau, 2001).

Phytophthora brown rot

Introduction

Phytophthora brown rot generally occurs when rainfall is high at the late stages of fruit development and ripening (Savita and Avinash, 2012; Gade and Lad, 2018). It can be of economic importance on all citrus types but can be very severe on lemons (Graham and Menge; 2000; Adaskaveg *et al*., 2015). It is furthermore stated that the importance of a pathogen is associated with the time of year it causes damage. For example, some species like *P. citrophthora* are more prone to be the causal agent of brown rot in cooler temperatures while *P. nicotianae* are more prevalent in warmer climates. Early maturing citrus types are more susceptible to brown rot infection than late maturing cultivars. Brown rot are mainly a postharvest problem and are usually localized and can occur annually under favourable conditions (Adaskaveg *et al*., 2015).

Etiology

Brown rot can be caused by *P. palmivora* in more tropical areas. *P. citrophthora* and *P. nicotianae* are more prevalent in Mediterranean areas. *P. hibernalis* and *P. syringae* are also associated with brown rot but not as frequently (Graham *et al.,* 1998; Alvarez *et al*., 2008; Adaskaveg *et al*., 2015). *P. citrophthora* are considered to be the main cause of brown rot but *P. palmivora* is seen as being more aggressive. Both pathogens can infect fruit throughout the canopy by means of wind and/or rain dispersal from the soil. Both produces sporangia more rapidly on fruit which leads to rot and severe losses of fruit (Del Rio *et al*., 2004). *P. nicotianae* produces less sporangia on the surface of the fruit, as less infecting propagules are available to be splashed from the soil higher into the canopy to infect the fruit and subsequently regarded as a minor brown rot pathogen (Graham *et al*., 1998; Timmer *et al.*, 2000). Nonetheless, *P. nicotianae* is regarded as the predominant *Phytophthora* spp. in South Africa (Meitz-Hopkins *et al.*, 2014), causing brown rot of citrus fruits. It was recently found that brown rot is also caused by *P. sp. prodigiosa* and *P. sp. mekongensis* in Vietnam on pomelo, and showed pathogenicity towards bergamot, grapefruit and sweet orange (Puglisi *et al*., 2017).

Symptoms

Usually the mature fruit on the lower part of the canopy are infected when infecting propagules are splashed up from the soil, or the fruit come in direct contact with soil surfaces (Gade and Lad, 2018). The first symptoms that are visible on the rind is decay that starts as a circular

light brown or olive discolouration that gives the fruit a leathery appearance (Tashiro *et al.*, 2012). The area that are infected remains firm with a very characteristic odour but does not develop sunken lesions (Graham and Menge, 2000). At high humidity, white mycelia can develop and cover the surface of the decaying area on the fruit (Adaskaveg *et al*., 2015). The fruit can eventually mummify and drop (Erwin and Ribeiro, 1996). Those that do not show symptoms are picked and taken to cold storage where the symptoms develop later during transport, storage and possibly in the market. All cultivars are susceptible to brown rot, but lemons are shown to be most sensitive (Graham and Menge, 2000).

Epidemiology and disease cycle of *Phytophthora*

Phytophthora is a soilborne pathogen which mainly lives and reproduces in the soil (Gade and Lad, 2018). Populations persist in the soil by continuously infecting fibrous roots of host plants and are favoured when climatic conditions are optimal and enough susceptible host tissue for infection is available (Graham and Menge, 2000). Inoculum sources are important for the disease cycle and free water is needed for inoculum build up and distribution to occur (Adaskaveg *et al*., 2015). When moisture and temperature levels are favourable to *Phytophthora* spp. prevalent in a region, it produces sporangia which in turn produce and releases motile zoospores in the soil (Matheron and Matejka, 1992; Graham and Menge, 2000; Oren and Yogev, 2002).

New roots release natural exudates from the elongation zone into the surroundings and zoospores are attracted by these naturally released nutrients, encyst, and germinate when contact with the roots occur (Graham and Menge, 2000). This is followed by infection of the elongation zone and cortex and eventually the whole rootlet will rot (Graham and Menge, 2000), leading to Phytophthora root rot. Rain that splashes soil containing infectious propagules to the parts above the soil surface, below or above the bud union, is also an important way of distribution (Alvarez *et al.*, 2008). A study conducted in 2009 showed that snails, *Helix aspersa*, could be a possible vector of *Phytophthora* spp. infectious propagules from the soil to higher up the trunk. If there is a natural opening in the bark or bark cracks and wounds, infection can also take place through these openings (Savita and Avinash, 2012). If the cambium under the bark is exposed, it can be susceptible to infection for approximately 14 days (Graham and Menge, 2000).

Gumming of the trunk and foot rot are a result of this infection but do not commonly produce additional inoculum and therefore has no further role in the epidemiology. When infection starts through natural openings in the bark or wounds, combined with moisture levels that are fairly high, *Phytophthora* spp. colonizes the cambium tissue and later the phloem (Vicent *et al.*, 2012). This leads to the development of branch cankers. As crop maturity and temperature play a pivotal role in branch canker development, a seasonal infection pattern

was noticed (Alvarez *et al.*, 2009). Generally, bark infections on citrus trees and lesions occur in spring and autumn, specifically for Mediterranean climates, when the temperatures are mild and favour lesion development (Alvarez *et al*., 2009).

Brown rot of fruit in the orchard occur when *Phytophthora* spp. are splashed up from the soil, that contains the sporangia or zoospores, to the mature fruit and foliage in the canopy (Timmer *et al.*, 2000; Oren and Yogev, 2002; Graham and Feichtenberger, 2015). Brown rot therefore develops faster in rainy conditions and the more the fruit matures, the more susceptible it becomes (Erwin and Ribeiro, 1996). Brown rot fruit losses differ from year to year due to variable weather conditions playing a role in disease development (Graham and Feichtenberger, 2015). Some infected fruit will fall from the tree but some harbours latent infections that can result in decay while in storage (nesting effect) due to secondary pathogen infections by *Geotrichum* and *Penicillium* spp. (Erwin and Ribeiro, 1996; Savita and Avinash, 2012; Tashiro *et al*., 2012; Adaskaveg *et al.*, 2015; Graham and Feichtenberger, 2015). Brown rot can develop into epidemics when areas are exposed to wetting periods of longer than 7 days during the late stages of fruit ripening and maturation (Graham *et al.*, 1998).

If trees are cultivated in compacted soil with little drainage and soil are heaped against the trunk it can aid in the infection, dispersal and survival of *Phytophthora* spp. (Erwin and Ribeiro, 1996; Alvarez *et al.*, 2008). Stagnant water that remain in contact with the trunk for as little as five hours also promote infection. Furthermore, if the soil pH is around 6, and soil is extensively wet, it also favours disease development (Erwin and Ribeiro, 1996). *Phytophthora* spp. has the capability of surviving in root debris for extended periods of time with the formation of chlamydospores that are produced when the climatic conditions are not favourable (Graham and Menge, 2000; Savita and Avinash, 2012). When the conditions are favourable, these resting spores will germinate and the cycle continues accordingly (Savita and Avinash, 2012).

MANAGEMENT OF *PHYTOPHTHORA* DISEASES ON CITRUS

Pre-harvest management

Cultural

Cultural practices should be considered as highly important and should be altered if need be, as the environment is ever changing (Alvarez *et al.*, 2008). Main precautions, aimed at preventing infections, should always be in place when working in citrus nurseries or orchards. In field nurseries, greenhouses and if an orchard is being established, the risk of *Phytophthora* contamination can be minimized by taking basic precautions such as planting certified, disease free material (Savita and Avinash, 2012). Citrus fruit that has fallen should be

discarded. Seeds can be heat treated before planting with 50°C for up to 10 min to make sure that *Phytophthora* spp. will not spread by these means (Graham and Menge, 2000; Savita and Avinash, 2012). If a planting site are left uncultivated for at least six months to a year, all the roots and debris will decompose and effectively lower the inoculum levels. The use of solarization (soil hydrothermal heating) can speed up the process. This can be done by covering the planting row using a translucent polyethylene sheet in the hottest months of the year for approximately nine weeks (Le Roux, 2009).

In nurseries, there are usually several problems at the seedbed stage, which can result in the damping-off of the seedlings. This can be avoided if the seeds are planted indoors (shade houses or greenhouses), in sterile containers and with the use of in sterile media without soil, which reduce the risk factor of the seedbed stage (Graham and Menge, 2000). Individuals working within a specific site should regularly sanitize hands, shoes (footbaths), equipment (steam cleaned and dried) and vehicles (wheels baths) (Graham and Menge, 2000). To ensure that the susceptible scion does not get infected in the nursery, budding high on the rootstock seedling should be done (Graham and Feichtenberger, 2015).

Resistance of rootstocks can be considered as the best management strategy for *Phytophthora* diseases (Graham and Feichtenberger, 2015), especially managing foot and root rot. Rootstocks can differ significantly in susceptibility to *Phytophthora* spp. (Graham and Menge, 2000; Graham and Feichtenberger, 2015). The choice of rootstocks are usually based on several characteristics of the orchard such as the water (irrigation system and water quality), soil characteristics (pH, texture, clay content, nutrient status, salt- and moisture levels), history, status of soilborne diseases and micro- and macroclimate characteristics (Lee *et al*., 2009; Graham and Feichtenberger, 2015). Rootstocks are defined as resistant when infection of the roots occur, but do not rot. If the soil are infested with *Phytophthora,* roots can be considered tolerant because it generates new roots to maintain root density (Graham and Feichtenberger, 2015).

Le Roux (2009) reports that sweet orange, volkameriana lemon and rough lemon are highly susceptible to *Phytophthora* spp., and Cleopatra mandarin (*C. reticulata*) and Sun Chu Sha mandarin are susceptible. Intermediate susceptible rootstocks include Minneola x trifoliate hybrid [(*C. paradise x C reticula*) x *P. trifoliata*], Carrizo-, Troyer-, Yuma- and C32 citrange and X639 hybrid (*P. trifoliata x C. reticulata*). The rootstocks tolerant to *Phytophthora* spp. are Rusk-, C35- (*C. sinensis x P. trifoliata*) and Benton citrange, Swingle citrumelo (*C. paradisi x P. trifoliata*), sour orange (*C. jambhiri*) and Trifoliate orange (*Poncirus trifoliata*) (Le Roux, 2009). Graham and Feichtenberger (2015) states that some rootstocks are specifically susceptible, tolerant or resistant to a species of *Phytophthora*; for instance, sour orange and Cleopatra mandarin are susceptible to *P. nicotianae* that causes root rot, but trifoliate orange and its hybrids are tolerant to this pathogen. In addition, Cleopatra mandarin and sour orange

are tolerant to *P. palmivora* but Carrizo citrange, Swingle citrumelo and trifoliate orange are susceptible to this species.

It is important to keep in mind that the susceptibility could change under certain environmental conditions and even if other selections are budded on the same rootstock (Le Roux, 2009). These tolerant rootstocks are resistant to infection by the citrus bark and root rot pathogens (Graham and Menge, 2000). It is further stated that the tolerance of the rootstock, which leads to tolerance to root rot, are due to factors that has to do with the biochemical resistance that improve the root health, regeneration, and decreases the pathogen access to the roots. In Morocco, the highest densities of *Phytophthora* spp. propagules were found when *Citrus volkameriana, Sunki mandarin* and *Carrizo citrange* rootstocks were used and associated with Valencia Late in a 17-year-old orchard. In contrast, the lowest density propagules occurred when *Citrus aurantium, Citrus macrophylla, Poncirus trifoliata,* Cleopatra mandarin*,* citrumelo 4475 and Goutou were used (Boudoudou *et al*., 2016).

Monitoring is very important to know if *Phytophthora* spp. propagules are present in the soil or not. If this oomycete is detected, it is important to establish whether or not the population is high enough to be damaging (Graham and Feichtenberger, 2015). Qualitative detection using leaf- and fruit baiting is fairly simple and minimum supplies are needed (Graham and Feichtenberger, 2015). Furthermore, selective media are used for *Phytophthora* spp. isolations to determine the propagule density in the soil, which serves as a quantitative measurement. The density will be at its highest closest to the roots and routine sampling should therefore take place when citrus trees look healthy and not diseased as *Phytophthora* infested trees have minimal roots, thus few propagules. The exact damaging *Phytophthora* population has not been established, but is estimated as 15 propagules/ cm^3 (Graham and Menge, 2000; Graham and Feichtenberger, 2015).

It is more difficult to detect damage of the roots in older orchards. Phytophthora root rot should rather be prevented as chemical management are usually not effective at controlling this disease. *Phytophthora* spp. can infect fibrous roots in a matter of hours, when the environment is favourable, and can kill the roots in four to six weeks. The loss of fibrous roots can lead to a lower yield and tree decline (Graham and Feichtenberger, 2015). The roots can also get damaged when soil depth is limited due to compacted soil layers and high water tables that are present (Graham and Menge, 2000).

Management of foot rot and gummosis should mainly be focussing on preventative measures and it is important to note that all scions' cultivars and rootstocks of citrus trees are susceptible if the environmental conditions are favourable (Graham and Menge, 2000). Creating optimal conditions for the pathogen should be avoided. For example, avoid wrapping the tree for both protection against the cold and to control sprouting of the trunk (Graham and Menge, 2000). However, a ring can be placed approximately 45 cm away from the trunk to

keep the stagnant water away. Other preventative measures that can be taken to control foot rot and gummosis is to plant trees with the bud union relatively high above the soil level (Graham and Feichtenberger, 2015).

For both root and foot rot control, the irrigation should be properly managed and adequate drainage of the soil is essential. Proper drainage and irrigation systems must be in place and water must not stand and stagnate in orchards because the pathogen's population can build up in the soil that leads to infection of the roots (Graham and Menge, 2000). Wetness period can be reduced by irrigating in the morning and late afternoons to let the tree dry naturally. Furthermore, irrigation water should not come in direct contact with the tree trunk as the constant wetness of the bark can lead to development of cankers (Graham and Menge, 2000). The ideal irrigation of a citrus tree is that the top 60 to 90 cm of soil should be wetted while the top 30 cm must be allowed to dry before irrigation is done again. The period of drying aids in the regeneration of the roots as it improves soil temperature and aeration. This has a negative effect on oomycete propagules present in the soil (Graham and Feichtenberger, 2015). Irrigation water should be tested frequently and treated if infested with *Phytophthora* spp. (Graham and Menge, 2000).

No debris or weeds should be under the trees and to avoid any unnecessary entry points for the pathogen, there should be no wounding of the bark. It is also important to control insects so that they do not feed on the wet bark and contribute to the wounding of the trunk. Infection areas and other wounds could also be the result of mechanical injuries or even very strong winds (Le Roux, 2009; Savita and Avinash, 2012).

It is important to minimize inoculum levels in the soil before the season starts (Graham and Feichtenberger, 2015). Brown rot severity is reduced if the period of wetness is minimized (Graham and Menge, 2000). To ensure that orchard wetness periods are minimized, certain preventative measures can be applied. This includes 1) good soil drainage and effective management of the irrigation system, 2) removing weeds by mowing around the trees and pruning of branches for maximum sunlight penetration and air flow (Graham and Menge, 2000) and 3) removing low hanging branches where most of the infections of fruit occur (Adaskaveg *et al.*, 2015). Harvesting of low hanging fruit for export should be avoided. Alternatively, these lower hanging fruit can be distributed to the local market (Adaskaveg *et al.*, 2015). Fruit for export should only be harvested 50 cm or more from the soil surface. When producers know that fruit infection has potentially taken place, and to prevent infected fruit from reaching the pack house, harvesting can be delayed allowing all infected fruit to fall to the ground (Graham and Menge, 2000). The recommended temperature of fruit storage is at 5 to 7°C for the control of postharvest brown rot (Timmer *et al.*, 2000) as it delays the infection development.

For organic and sustainable agriculture, alternatives must also be investigated, leading to the interest in the control of *Phytophthora* spp. with biological agents. In studies it was shown

that *P. parasitica* was effectively supressed *in vitro* by *Trichoderma virens* and *T. harzianum* (Gade and Lad, 2018). Additionally, *Pseudomonas* spp. produced siderophores that were shown to control *Phytophthora* diseases in glasshouse trials and increased growth of small citrus trees. *P. fluorescence* strain Pf IV was linked to the reduction of mycelial growth of *P. parasitica*. Gummosis and root rot can effectively be managed when *P. fluorescence* are used in combination with certain fungicides on mandarin. All the *Phytophthora* spp. that were tested *in vitro*, were effectively inhibited by *Trichoderma viride* and *Chaetomium globosum, Gliocladium virens*, including *P. nicotianae*. In addition, *Bacillus* spp. also showed to supress *Phytophthora* root diseases in greenhouse studies (Gade and Lad, 2018).

Chemical

Fungicides will always remain an important part of integrated management strategies to maintain healthy crops, resulting in higher yields (Brent and Hollomon, 2007). Chemical control is an important aspect in managing *Phytophthora* diseases of citrus. Fungicides used include fosetyl-Al, phosphite salts, mefenoxam and copper products (Dewdney *et al.,* 2019). It is important to know the biochemical and physical mode of action of chemical compounds (Matheron and Porchas, 2000). The value of the chemical compound lies with the ability to control a disease at the physiological level of the pathogen, targeting either one or more life cycle stages (Matheron and Porchas, 2000). Furthermore, the highest risk of infection and disease development occurs at the life cycle stages where the pathogen build up infecting propagules, such as the zoospore release- and sporangium formation stage in the case of *Phytophthora* spp.

Thus, any chemical that can reduce the motility of zoospores, inhibit the formation of sporangia or encysting zoospores, should in theory reduce *Phytophthora* diseases. *Phytophthora* levels in the soils should be monitored seasonally to determine if the levels are damaging in a specific season. Usually fungicides are recommended when the propagules are 10 to 20/g or cm^2. These levels can become harmful and can increase when the wetness increases (Graham and Menge, 2000). In recent years several foliar- and soil treatments were registered such as phosphonates (calcium- and potassium phosphite and fosetyl-Al) and soil treatments such as phenylamides (metalaxyl/mefenoxam) (Adaskaveg *et al.,* 2015) for the management of *Phytophthora* diseases. It is important to alternate between fungicide chemical classes to avoid the chance of resistance development within *Phytophthora* populations. Fungicide resistance is a serious problem and the overuse of any fungicide should always be avoided as fungicides in general should not be a substitute for basic sanitation (Brent and Hollomon, 2007).

Chemicals can be used to control foot rot in young trees if cultural control is inadequate. Three things are important to establish prior to applying any post planting chemicals namely,

the probability of *Phytophthora* spp. infection occurring at the nursery stage, susceptibility of the rootstocks and the presence of previous *Phytophthora* diseases in that area (Graham and Feichtenberger, 2015). The post planting fungicides include chemicals with phosphite (PO_3^{3-}) or metalaxyl as active ingredients. These can be applied as foliar sprays, soil drenches or trunk paints. These fungicides are systemic and are usually regarded as very toxic to *Phytophthora* spp. (Erwin and Ribeiro, 1996). A spray program can be followed for the first growing season if the rootstocks are tolerant but can be continued if the rootstocks are susceptible (Graham and Feichtenberger, 2015).

Contact fungicides, such as captan, cupric hydroxide, Bordeaux mixture, copper ammonium carbonate, captafol and dithiocarbomates such as manzate and zineb, can be used to protect the trees when these fungicides are being applied to the trunk or base of the tree. However, none of these compounds can eradicate *Phytophthora* infections (Erwin and Ribeiro, 1996). In the past, the control of foot rot consisted of a copper paste that was placed on the lesions. However, as this is a contact fungicide, it did not provide protection of roots. In general, contact fungicides are used as a preventative measure at specific sites where there is a chance that some cultural practices may harm the tree (Le Roux, 2009).

Phosphonates are regarded as relatively inexpensive fungicides (Graham and Feichtenberger, 2015). Phosphorous in its naturally occurring oxidised form is phosphate (PO_4^{3-}), and phosphite has one less oxygen atom (Graham, 2011). When phosphite is sprayed on citrus foliage, the chemical moves into leaves in a matter of a few hours and is translocated through the xylem to the roots within a few days (Rebollar-Alviter *et al*., 2010; Graham, 2011). Phosphite can also move from the leaves to the fruit through the phloem (Graham, 2011) and protect the plant below and above the soil when being applied at least 35-40 cm above the soil level (Le Roux, 2009). It was found that phosphite gives protection through a unique mode of action that includes indirect and direct defence responses of the host plant itself. A plant can recognize *Phytophthora* spp. infections and activate a pathway called phenylpropanoid, which in turn synthesise defence compounds such as lignin and phytoalexins (Graham, 2011). Thus, when phosphite is applied, the chemical has a reaction where the pathogen gets a phosphate shortage and excrete stress metabolites that induce even higher defence mechanisms in the plant.

Fungicides are assigned a group depending on their mode of action. This is done by the Fungicide Resistance Action Committee (FRAC). Mefenoxam (Metalaxyl-M), chemically similar to metalaxyl, is a good preventative fungicide, and has curative activity as well (Rebollar-Alviter *et al*., 2010). It consists of a single-site mode of action, which makes resistance within a pathogen population a great risk (Porter *et al*., 2009). Given its mode of action, it belongs to FRAC group 4. It inhibits the ribosomal RNA polymerase, thereby inhibiting sporulation and growth of mycelium (Hu and Li, 2014). From the 1990's, mefenoxam

and metalaxyl resistance was found to be common in populations of *P. nicotianae* from citrus, as well as other plant species (Panabières *et al*., 2016).

For the management of root rot, it is important to establish if the losses of fibrous roots in mature orchards are due to wet conditions. If that is not the case, then *Phytophthora* populations should be monitored before and after chemical treatment (Graham and Feichtenberger, 2015). The chemical control program must be based on seasonal applications during susceptible root flushes and should be applied directly at the highest density area of the roots (Graham and Feichtenberger, 2015). For root rot control, there are acylalanines (Ridomil Gold) and phosphonate (Aliette WP, Phytex and Phytofos) chemical groups. The acylalanines are used for soil applications to control *Phytophthora* in the soil as well as in the roots and the phosphonates is registered as foliar sprays and trunk paints (Le Roux, 2009; Savita and Avinash, 2012). Injecting the stem with phosphonates was never a good option with citrus growers as it left a wound that did not heal fast enough, subsequently becoming infected with secondary pathogens (Le Roux, 2009).

There have been reports of *Phytophthora* resistance towards mefenoxam and potassium phoshite, due to the fungicides being on the market for an extended period. Based on this fact, greenhouse and field studies were done using four alternative compounds with different modes of actions against root rot of citrus (Hao *et al.,* 2019). These compounds were mandipropamid, fluopicolide, oxathiapiprolin and ethaboxam. Following this preharvest study, mandipropamid and ethaboxam are in the registration process and oxathiapiprolin has received full registration on citrus. Fluopicolide furthermore has federal registration in the USA (Hao *et al*., 2019).

Trunk and branch canker control relies on being vigilant for gumming, indicating infection, on the scion and branches of citrus trees as it only occurs superficially under the bark. If detected early, a thorough spray of the trunk and branches with a mixture of didecyldimethyl-ammonium chloride (DDAC) and the contact fungicide, captan, gives very good control of this disease (Tian Schutte, pers. comm.).

Originally brown rot was chemically field treated just before rainy periods in harvest season with neutral or fixed coppers (oxide or hydroxide) in mixture with hydrated lime or Bordeaux mixtures (copper (II) sulfate and slaked lime) (Oren and Solel, 1978). Currently, for the management of brown rot, adequate protection is usually provided with a single application of phosphite before signs of brown rot are visible in the field. This can provide up to 90 days of control and give protective action toward postharvest infection, but are not as effective when applied pre-infection (Graham and Feichtenberger, 2015). Copper fungicides can be effective in eliminating viable propagules that are present on fruit surfaces. These can still be applied after the symptoms of brown rot appeared and can provide protection for up to 60 days. For pre-infection protection of fruit, it is effective to apply copper fungicides before it rains. Copper

is a protectant contact fungicide which has a multi-site activity and the probability of resistance is unlikely. At high temperatures it can be phytotoxic which limits its use. As copper is a contact fungicide, it has to be applied often because the leaves and fruit grow out of protection (Vega *et al*., 2012).

Postharvest management

Brown rot control should follow an integrated strategy to prevent this rot from spreading to other healthy citrus fruits in storage. This can lead to decay anywhere in the supply chain namely cold storage, transportation and when on the market (Adaskaveg *et al.,* 2015). Thus, any potential postharvest treatment should have the ability to prevent possible decay when fruit were infected in the orchard shortly before harvest (Adaskaveg and Förster, 2014).

Metalaxyl has postharvest efficacy for *Phytophthora* control, where it is used as an in-wax application before packing for shipment. Postharvest dips of metalaxyl or fosetyl-Al can prevent the spread of *Phytophthora* in packing boxes for up to 60 days if it is kept at 11°C (Erwin and Ribeiro, 1996). *Penicillium* rots were not managed with these treatments, but in combination with imazalil or thiabendazole, both rots could be managed. Previously, heat treatments in combination with fungicides were also used. However, there is always the danger of the fruit being damaged during the process. This makes the fruit even more susceptible to secondary decay pathogens (Erwin and Ribeiro, 1996).

Azoxystrobin, fluopicolide, mandipropamid and potassium phosphite was tested as possible postharvest treatments for the control of brown rot and all four were very effective when applied as protective treatments. Only potassium phosphite exhibited curative abilities (Adaskaveg and Förster, 2014). In the USA, Adaskaveg *et al*. (2015) registered potassium phosphite in combination with heat treatments for the postharvest management of Phytophthora brown rot. However, exposure times and fruit temperatures should be carefully monitored. In South Africa, heat treatments are not a sustainable option for the control of Phytophthora brown rot as these treatments make the fruit more susceptible to cold damage during export. Cold damage can specifically occur during cold sterilization that is done during export to the EU for the control of false coddling moth (FCM). Ramallo *et al* (2019) also did an application of potassium phosphite before and after artificial inoculations of *Phytophthora* on lemons and concluded that the post-harvest applications had moderate curative abilities, but the protective abilities were significantly better when applied one week before infection.

Preharvest phosphonates are being used extensively because of the ease of application, lower costs, easy translocation within trees and the wide-spread resistance of *Phytophthora* spp. to phenylamide fungicides (Adaskaveg *et al*., 2017). With the extensive use of these fungicides, a higher probability of resistance tends to develop within a pathogen population. Nonetheless, there have been several reports of resistance towards phosphonates

(Adaskaveg *et al*., 2017). However, due to limitations of phosphorous acid residues on fruit to certain export markets, its extensive use has been prohibited. This led to other options for postharvest control of brown rot being investigated (Adaskaveg *et al.,* 2015; Hardman and Hattingh, 2015).

Applications of ametoctradin and dimethomorph were reported to reduce growth of *Phytophthora* spp. (Merk *et al*., 2011). These substances were recently tested against *P. citrophthora* to control brown rot on lemons (Ramallo *et al*, 2019). Ametoctradin and dimethomorph in combination inhibited the sporangia formation, mycelial growth and, influenced the zoospore's motility and structures (Ramallo *et al*, 2019). The postharvest application of these substances in combination had good protective action but did not have curative abilities (Ramallo *et al*, 2019).

There is currently no postharvest treatments registered for the control of Phytophthora brown rot in South Africa and the two main species found in citrus orchards are *P. nicotianae* and *P. citrophthora.* Both have been shown to be involved with brown rot on fruit, not only in South Africa, but also other citrus production areas such as Florida and California (Graham *et al*., 1998; Timmer *et al*., 2000; Meitz-Hopkins *et al*. 2014).

Azoxystrobin and fludioxonil are two fungicides that have been investigated for their ability to control green mould (*Penicillium digitatum*) due to resistance development within *P. digitatum* isolates to imazalil, thiabendazole and other traditional postharvest fungicides (Kanetis *et al*., 2007; Schirra *et al*., 2010; D'Aquino *et al*., 2013). Both were found to give good control of imazalil resistant and sensitive isolates of *P. digitatum* (Kanetis *et al*., 2007; Schirra *et al*., 2010; D'Aquino *et al*., 2013). Adaskaveg and Förster (2015) showed that azoxystrobin gave good pre-infection control of Valencia or Navel orange fruit to Phytophthora brown rot. However, they only looked at inoculating fruit with *P. citrophthora* either 15 hr before treatment or 6 hr after treatment. Azoxystrobin was registered in South Africa as postharvest treatment of citrus to control *Penicillium* spp in May 2019, (Suzel Serfontein, pers. comm.).

Azoxystrobin is a systemic fungicide, falling into the group of quinone outside inhibitor (QoI) fungicides that interferes with the mitochondrial respiration of the fungus. This activity results in binding at the complex III of cytochrome *bc1* (ubiquinol oxidase) at the Qo site, which is the cyt b gene, in the mitochondrion of the electron transport chain. In other words, it prevents transfer of electrons between cytochrome b and c (Anand *et al*., 2008; Vega *et al*., 2012). This eventually results in the prevention of the production of adenosine triphosphate (ATP). Furthermore, Anand *et al.* (2008) states that azoxystrobin has a broad spectrum of control which includes Oomycetes, Deuteromycetes, Basidiomycetes and Ascomycetes and has relatively high activity levels at low rates. This fungicide has vaporising-, translaminar- and protective activities at the post symptom- and post infection stage of disease development

(Anand *et al.*, 2008). This mode of action makes it effective against fungi which are less sensitive to other fungicides (Anand *et al.*, 2008).

Azoxystrobin is also a different fungicide chemical class than phosphite and mefenoxam/metalaxyl and if these actives are used in an alternating fashion, it could inhibit or delay fungicide resistance (Rebollar-Alviter *et al*., 2010). QoI fungicides, and other fungicides with a specific site of action, have an increased risk of fungicide resistance to a multi-site fungicide (Sozzi and Staub, 1987; Vega *et al.*, 2012). Thus, azoxystrobin (methoxy-acrylates, FRAC group 11) has a high risk of resistance. There has been reports of azoxystrobin resistance in some fungal pathogens such as *Cercospora beticola, Pyricularia oryzae, Zymoseptoria tritici* and *Colletotrichum siamense* as well as resistance towards azoxystrobin in *P. capsici* (Ma *et al*., 2018).

Imazalil, thiabendazole, pyrimethanil and fludioxonil are regarded as citrus postharvest fungicides (Smilanick *et al*., 2008). When potassium sorbate was added to the fungicides combined with heat, the result was that it improved the control of postharvest pathogens, *Geotrichum citri-aurantii*, and *P. digitatum.* Fludioxonil is a non-systemic fungicide that influence the pathogen's signal transduction. This fungicide belongs to the phenylpyrroles chemical class and is a derivative of an antibiotic produced by *Pseudomonas* bacteria (Gao *et al*., 2018). It interferes with osmotic signal transduction, MAP/ histidine-kinase (os-2, HOG1) and therefore inhibit the associated transport phosphorylation of glucose, which reduces mycelial growth. Fludioxonil is considered as a broad spectrum fungicide against plant pathogens (Errampalli, 2004) and is already registered for postharvest use on citrus in South Africa. It was specifically registered as application in wax aimed at sporulation inhibition of *Penicillium* spp. (Du Plooy and Lesar, 2017). Fludioxonil (phenylpyrroles, FRAC group 12) is regarded as having a medium to high resistance risk.

However, Adaskaveg and Förster (2015) states that as of yet no practical resistance has occurred to azoxystrobin and fludioxonil in the postharvest pathogen *P. digitatum.* Resistance has only occurred in the laboratory for azoxystrobin and only in air sampling in packhouses as well as selective media in laboratories for fludioxonil. It is also yet to be seen if azoxystrobin and fludioxonil applied in wax can have any curative effect on postharvest brown rot infections and to protect the fruit against the spread of brown rot during storage and transit.

FUNGICIDE SENSITIVITY TESTING

The baseline sensitiviy of a pathogen population towards a fungicide is important to eshtablish before the introduction of the chemical to the market (Brent and Hollomon, 2007). This baseline sensitivity information is essential for monitoring development of possible resistance to a fungicide in pathogen populations. Detection of resistance will allow management of that resistance. There are various methods of testing the sensitivity of fungal pathogens towards

different concentrations of fungicides. Methods used to test sensitivity are either more traditional techniques or automated, quantitative methods. With both traditional and modern techniques, there are certain advantages and disadvantages.

A traditional and more conventional method which test sensitivity towards fungicides, is to amend molten media with different concentrations and measure it as parts per million (ppm) of the fungicide. The petri dishes are inoculated with the pathogen and after a specific amount of time, mycelial growth inhibition can be determined by measuring the colony diameter perpendicularly. The percentage inhibition for each plate at each concentration is calculated in relation to the growth on unamended plates and the EC (effective concentration) 50, 80 and 90 values can be determined (Frąc *et al*., 2016). The disadvantages of this more traditional method include that it is labour-intensive, time consuming and in continuous need of substantial space for plates and the large amount of media that must be prepared tend to get expensive. Measuring of the mycelial growth can be subjective from person to person. There is also the probability of the high amount of toxic chemicals that could be environmentally harmful or harmful to the person who are testing it (Vega *et al.*, 2012; Frąc *et al*., 2016).

The development of more modern techniques is usually limited by the specialized and costly equipment that are needed. Micro-assays have been developed where the growth of the fungus are measured optically in microtiter plates (Förster *et al*., 2004). Colorimetric and bioluminescence tests use reagents that are toxic to humans and the organisms being tested. With the use of resazurin, which is a safe, stable, non-toxic, water-soluble, tetrazolium-based dye, it has more of a long-term use. The non- fluorescent and oxidised state are blue and if cell metabolism occurs, it fluoresces pink. Thus, this dye can be spectrophotometrically and fluorometrically measured (Vega *et al.,* 2012). This technique requires less supplies, but specific equipment, and is still labour intensive when pipetting at various stages (Förster *et al*., 2004). This assay is also limited if hydrophobic compounds are tested, due to precipitation interference with the density measurements (Förster *et al*., 2004). In a study conducted by Frąc *et al.* (2016) MT2 microplates were used which were originally for the identification of bacteria. In this study, it was used as an alternative for detecting *Fusarium* fungicide resistance. With the use of azoxystrobin, carbendazim and tebuconazole at different concentrations with different isolates, they concluded that this method is time saving and effective, compared to the traditional petri dish approach.

Bioassay techniques requires similar plant tissue to be readily available. This technique consists of the infection of plant tissue with the specific pathogen and subsequently being treated with various fungicide concentrations or firstly treated with fungicides and then infected, followed by evaluation (Förster *et al*., 2004). In 1987, Sozzi and Staub, tested the sensitivity of *P. infestans* isolates to metalaxyl on whole plants, leaves and potato leaf discs. Minor differences were observed in the three different methods of sensitivity testing. The one

major disadvantage from all three methods was that they were not quantitative and resistance lower than 0.1% remained undetected. In addition, these methods could only detect resistance at higher sporangia levels.

Another technique, the spiral gradient dilution (SGD), also originally developed for studies of antimicrobial compounds against bacteria, was introduced in the 1990's. It proved effective for fungicides against fungi (Förster *et al*., 2004). PDA was poured into petri dishes to form a specific layer, allowed to solidify, and used approximately 24 hrs later. The fungicides were spirally applied (exponential application) to the plates with the use of a spiral plater. It was subsequently inoculated with cellophane strips covered with fungal mycelia, over the fungicide concentration gradient. After incubation, radial growth was measured (Förster *et al*., 2004). It was stated that this technique required less supplies and is faster than traditional techniques and that the software is user friendly. In contrast, this method could get very expensive if no access to a spiral plater is available and the SGD software needs to be purchased.

Numerous other techniques have been developed for QoI resistant genotypes detection.

These techniques involve polymerase chain reaction (PCR) technologies and the allele-specific PCR or CAPS-PCR. These are generally versatile, low cost and straightforward with no special PCR equipment needed to identify fungal mutants that are resistant. In contrast, using only PCR based methods to detect fungicide resistant isolates, only detects resistance according to the PCR primers that are designed to detect resistant isolates. This technique cannot detect other possible causes of fungicide resistance. Another disadvantage is that there could also be silent mutations that do not change the phenotype, and this can lead to false negatives.

Quinone outside inhibitors have a relative high development resistance risk for pathogens due to having only a single site mode of action (Sierotzki et al., 2000). There have been resistance to QoI described in numerous fungi, and thus far, two mechanisms of resistance in pathogens have been identified (Piccirillo *et al*., 2018). The first resistance mechanism only happens *in vitro* and involve the alternative oxidase enzyme (AOX) which activates a shorter alternative pathway in the place of the usual respiration pathway and bypasses the complexes III and IV by transferring the electrons directly from ubiquinol to O_2 (Piccirillo *et al*., 2018). In the presence of QoI's, this mechanism is usually activated when normal respiration is inhibited. Therefore, the results of *in vitro* trials, to test for QoI fungicide sensitivity, can be inaccurate if an alternative respiration pathway is activated. That is why it is highly recommended to add AOX inhibitors, such as salicylhydroxamic acid (SHAM), with the QoI fungicide to block all possible *in vitro* alternative respiration pathways (Piccirillo *et al*., 2018). The other resistant mechanism involves substitution of specific amino acids which results in the prevention of effective fungicide binding (Piccirillo *et al*., 2018).

CONCLUSION

There are currently no postharvest fungicides registered in South Africa, thus Phytophthora brown rot management depends only on preharvest management strategies. Hence, there is a need for postharvest fungicides. In terms of postharvest Phytophthora brown rot management, very little is known about azoxystrobin, fludioxonil and potassium phosphite as potential postharvest control strategies and if these fungicides could provide some curative- or protective action. The aim of this study was therefor to evaluate these postharvest fungicides for Phytophthora brown rot control. The objectives were firstly to determine the *in vitro* efficacy of azoxystrobin and fludioxonil towards *P. nicotianae* isolates mycelial growth and to establish the potential of these fungicides. Additionally determine, along with potassium phosphite, their potential as aqueous postharvest dip applications for brown rot control and to determine if azoxystrobin and fludioxonil can limit brown rot infections from spreading within cartons (nesting effect).

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CHAPTER 2

In vitro efficacy of azoxystrobin and fludioxonil against *Phytophthora nicotianae* causing brown rot of citrus

ABSTRACT

Phytophthora brown rot caused by *Phytophthora nicotianae* can often cause serious postharvest losses. There are currently no postharvest fungicides registered for the control of this disease on citrus in South Africa. The objective of this research was therefore to evaluate the sensitivity of 111 isolates of previous unexposed and previous exposed populations of *P. nicotianae* towards azoxystrobin amended with SHAM, and fludioxonil *in vitro*. Results indicated that isolates from both previously unexposed and previously exposed pathogen populations could be divided into different azoxystrobin and fludioxonil sensitivity groups. These groups were statistically different based on their mean EC_{50} and EC_{90} values. For azoxystrobin, the EC_{50} values of the groups in the previously unexposed population ranged between 0.01 and 0.19 µg/ml and the EC_{90} values between 4.28 and 83.96 µg/ml. In the exposed population, the EC_{50} values for azoxystrobin was between 0.04 and 0.46 µg/ml and that of the EC_{90} values between 11.45 and 84.85 µg/ml. For fludioxonil sensitivity, the mean EC_{50} values of sensitivity groups in the previously unexposed population was between 5.56 and 1613.52 µg/ml, with the EC_{90} values of these groups ranging between 1988.50 and 9929.30 µg/ml. The means for the groups in the previously exposed population were similar. The highest EC_{50} value was 84.79 and the lowest 3.10 µg/ml. The highest EC_{90} was 6809.90 and the lowest 1090.50 µg/ml. This information indicates that no shift in sensitivity was seen from the baseline population to the non-baseline population for both fungicides.

INTRODUCTION

Oomycetes have very distinct characteristics separating them from true fungi (Meng *et al*., 2014). The genus *Phytophthora* often stands out because of the high number of species and hosts which has negative effects on agricultural production (Erwin and Ribeiro, 1996). One of the most prevalent *Phytophthora* spp. on citrus is *P. nicotianae* Breda de Haan because of its broad host range (255 plant genera, in more than 90 families). This species is seen as contributing to heavy losses and is present in multiple ecological niches (Panabieres *et al.,* 2016). On citrus, it causes foot and root rot as well as brown rot of the fruit (Savita and Avinash, 2012).

Brown rot has economic importance and usually occurs when rainfall is high at late stages of fruit ripening and development (Graham and Menge, 2000; Adaskaveg *et al*., 2015). The

risk of brown rot can be minimized with effective cultural practices such as skirting of trees and the removal of fruit that is hanging closest to the ground (Graham and Feichtenberger, 2015). Furthermore, a single application of phosphite can provide protection for up to 60 days as well as postharvest protection and marginally effective if applied after infection. Copper fungicides are able to kill infecting propagules on foliage or fruit surfaces (Graham and Feichtenberger, 2015). In South Africa, there are limited options for brown rot postharvest control. According to Adaskaveg *et al*. (2015) potassium phosphite was recently registered in the USA in combination with heat treatments for the postharvest management of Phytophthora brown rot. This is not a good option for South Africa because hot water treatments will make fruit more susceptible to cold storage damage during exports and SA needs cold storage during export to the EU for false coddling moth control. Thus, other possible fungicides should be investigated for the postharvest control of brown rot.

Due to resistance development of the citrus postharvest pathogen, *P. digitatum,* to imazalil, thiabendazole and other traditional postharvest fungicides, azoxystrobin and fludioxonil have been studied previously to specifically control this pathogen. (D'Aquino *et al*., 2013; Kanetis *et al*., 2007; Schirra *et al*., 2010). Both fungicides were found to give good control of *P. digitatum* resistant isolates to imazalil (D'Aquino *et al*., 2013; Kanetis *et al*., 2007; Schirra *et al*., 2010). However, azoxystrobin and fludioxonil have not been evaluated for their ability to control *P. nicotianae* and postharvest brown rot on citrus fruit. Both fungicides are regarded as new generation fungicides. New generation fungicides entail big developments in technology, selectivity, safer for regular usage, reduction of rates and effectiveness against specific diseases (Nabi *et al*., 2017). However, new generation fungicides have the tendency to consist of mode of actions only targeting a single site which could lead to the facilitation of resistance (Leadbeater, 2012).

Azoxystrobin belongs to the fungicide class methoxyacrylates, and is derived from natural strobilurins which are broad spectrum fungicides (Rebollar-Alviter *et al.,* 2007; Kanetis *et al.*, 2008). These fungicides (also known as quinone outside inhibitors- QoIs) interferes with the electron transport chain and subsequently inhibiting the pathogens mitochondrial respiration. This occurs through fungicide molecules blocking the transfer of electrons from cytochrome b and c1 (bc_1-complex) in complex III, which results in prevention of ATP production (Dave., 2002; Rebollar-Alviter *et al.,* 2007; Piccirillo *et al*., 2018). Three mechanisms of resistance have been identified in pathogens when exposed to strobilurins. The first mechanism of resistance is protein substitutions in the cytochrome b region, which can lead to full resistance or field resistance. The second mechanism occurs when the alternative oxidase enzyme (AOX) are expressed which give way to the use of an alternative respiration route. The last possible resistance mechanism is caused by transporters such as ATP binding cassette transporters (Rebollar-Alviter *et al.,* 2007). Therefore, when conducting *in vitro* fungicide

sensitivity trials, results can be inaccurate if an alternative respiration pathway is activated. It is therefore essential to add AOX inhibitors, such as salicylhydroxamic acid (SHAM), with the QoI fungicide to the amended medium, to block all possible respiration pathways (Piccirillo *et al*., 2018) and to get a more accurate representation of pathogen sensitivity towards the fungicide.

Fludioxonil belonging to the class benzodioxoles, in the family of phenylpyrroles, is a low to medium fungicide risk for the development of fungicide resistance (Gao *et al*., 2018). This fungicide is derived from pyrrolnitrin, which is an antibiotic, and are produced by *Pseudomonas* spp. (Rosslenbroich and Stuebler, 2000). Its mode of action towards a pathogen is the improper activation of the HOG/p38 MAPK (mitogen activated protein kinase) signal transduction pathway. This pathway facilitates the cell's adjustment to oxidative stress and high osmolarity (Randhawa *et al*., 2018). This leads to inhibition of mycelial growth, germination of spores and the elongation of the germ tube, and ultimately cell death (Rosslenbroich and Stuebler, 2000; Okada *et al*., 2005; Furukawa *et al*., 2012).

Fungicide resistance in pathogens is often a problem as mutations develop (single gene mutation or polygenic) during exposure to the fungicide. It is thus very important to monitor resistance or sensitivity to detect if that is the cause of a loss in disease control by a specific fungicide (Brent and Hollomon, 2007). The first step to monitor any resistance is to collect baseline data for a specific pathogen population before any commercialisation of a fungicide (Brent and Hollomon, 2007). Baseline sensitivity can be defined as sensitivity data of a previously unexposed population, or individual fungus, in response to a specific substance by using molecular and or biological techniques. Establishing baseline sensitivity as a reference point in a population is important in order to monitor if any resistance is developing. The sensitivity data of a population previously exposed to the active ingredient or substance can then be compared to the baseline data to see if shifts in sensitivity to a substance took place (Brent and Hollomon, 2007).

Due to the unknown ability of azoxystrobin and fludioxonil to inhibit the *in vitro* growth of *P. nicotianae,* causing brown rot of citrus fruits, as well as the unknown baseline sensitivity of this pathogen to these fungicides, the objectives of this study were as follows:

1. determine the efficacy of azoxystrobin and fludioxonil *in vitro* to inhibit the mycelial growth of *P. nicotianae*,
2. determine the baseline sensitivity of a previously unexposed *P. nicotianae* population to these two fungicides, and
3. determine the sensitivity of a *P. nicotianae* population with possible previous exposure to either of these fungicides or fungicides in the same fungicide groups.

MATERIALS AND METHODS

Collection of isolates

Phytophthora nicotianae isolates were collected from both organic and non-organic orchards. Twenty trees were randomly selected in a block of maximum 1000 trees. Isolates were obtained from soil samples taken under the citrus trees, halfway between the irrigation line and the trunk. Each sample weighed approximately 20 g and when combined, added up to 200 g (two large handfuls). Weeds, grasses and the top 2 cm of soil was removed before taking the samples. The samples were collected at a depth of approximately 20 cm, placed in a plastic bag, closed and kept away from direct sunlight. Soil baiting was done according to the technique of Grimm and Alexander (1973). This was done as follows: one teaspoon of soil was placed in each cubicle of an ice tray with one soil sample per ice tray. Sterile distilled water was added without overflowing of water between the cubicles. Two cubicles were used for the control (no soil added to the cubicles). Two unsprayed, washed citrus leaf discs of 0.5 cm^2 were left to float in each cubicle and covered with tin foil to prevent light infiltration. These covered ice trays were incubated for 2-3 days at ambient temperature in the laboratory. After incubation, four randomly selected leaf discs from each ice tray were plated out on selective PARPH media (Kannwischer and Mitchell, 1978), as well as the control leaves. Petri dishes was subsequently placed in the dark at 29°C for up to 3 days before being inspected for the presence of *Phytophthora* spp. colonies.

DNA extractions and identification

Following incubation, *Phytophthora* isolates were hyphal tipped from the PARPH dishes onto corn meal agar (CMA) (Sigma-Aldrich, St. Louis, Missouri, USA) to obtain pure cultures. These plates were incubated at 29°C for 7 days. The mycelia were scraped off the surface of the actively growing 7-day-old culture using a spatula or a scalpel and placed in a marked 2 ml Eppendorf tube. Six glass beads of 4 mm diameter (Lasec, Cape Town, South Africa) and 200 µl of 0.5M NaOH were added to the tubes and shaken in a Retsch shaker (Retsch, Haan, Germany) at 30 Hz for 5 min to lyse cells. Following the shaking step, the tube was centrifuged for 1 min at high speed. Five microliters of the supernatant were suspended in 495 µl of 100mM Tris-HCL (pH 8.0). From this DNA suspension, 2 µl were used in PCR reactions.

A 25 µl PCR reaction for one isolate consisted of 2 µl of above-mentioned genomic DNA, 12.5 µl Promega G2 GoTaq Master Mix, 0.75 µl of both primers (10 µM) ITS 4 and ITS 6 and 9 µl nuclease free water. Amplifications were conducted in a 2720 Applied Biosystems (Applied Biosystems, Foster City, California, USA) thermal cycler. This reaction amplified a portion of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA. The reaction parameters were as follows: 94°C, 5 min; 32 cycles of 94°C, 30 s; 58°C for 30 s, 72°C

for 30 s and completed with 72°C for 5 min and followed a final step at 4°C. PCR products were resolved in a 1.5% agarose gel and DNA fragments were visualized by staining with an ethidium bromide solution.

The resulting PCR products were restriction digested with enzyme *Hha*I according to the manufacturer's instructions (Fermentas Inc, Burlington, Ontario Canada). The single enzyme reaction mixture of one isolate consisted of 1 µl of the enzyme, 10 µl enzyme buffer (X10), 9 µl of distilled water and 8 µl PCR product. The 20 µl tubes were incubated at 37°C for 5-15 min. PCR-RFLP (restriction fragment length polymorphism) products were run on a 3% agarose gel and isolates with the same RFLP banding pattern were assigned to the same RFLP group. From each group, one or two isolates were sequenced and compared to GenBank (https://www.ncbi.nlm.nih.gov/) sequences to identify isolates to species level.

Fungicide sensitivity

In a pilot study to confirm that SHAM (99%; Sigma-Aldrich) was sufficient to block the alternative respiration pathway in *P. nicotianae* baseline and non-baseline populations, and to evaluate the effect on the growth of mycelium, 20 representative isolates (10 from each population) were tested using azoxystrobin [Obstructo, 25% active ingredient (a.i), suspension concentrate, ICA International Chemicals, South Africa] at 0-, 1-, 5-, 25-, 50-, 125-, and 500 µg/ml, with SHAM and without SHAM. For each azoxystrobin concentration, SHAM, dissolved in methanol, was added at 100 µg/ml (Rebollar-Alviter *et al*., 2007; Kanetis *et al*., 2008; Duan *et al*., 2012; Ma *et al*., 2018; Piccirillo *et al*., 2018). The total solvent never exceeded 1 ml in a litre and the solvent present in the media never inhibited the growth of mycelia. The pilot experiment was repeated once more.

Based on the pilot trial results, the final concentrations of azoxystrobin was adjusted to 0-, 0.25-, 0.5-, 1-, 10-, 100-, and 2000 µg/ml with the addition of 100 µg/ml SHAM for each concentration as described above. The molten amended CMA was poured in 90 cm petri dishes and when solidified, 5 mm diameter plugs were taken from the margins of actively growing cultures and plated out. The 0 µg/ml concentration plates for azoxystrobin contained no fungicide but was amended with SHAM. CMA was also amended with fludioxonil (Teacher, 23% a.i, suspension concentrate, ICA International Chemicals, South Africa) to final concentrations of 0-, 1-, 100-, 1000-, and 10 000 µg/ml. All inoculated, amended plates were incubated at 29°C for 7 days before measuring the colony diameter perpendicularly on each plate. For the baseline sensitivity, 51 isolates were used from organic orchards with no previous exposure to the fungicides or fungicides in the same fungicide groups, and for the non-baseline sensitivity, 60 isolates were used from orchards that had possible previous exposure to these fungicides or fungicides from the same fungicide groups. The same sets of isolates were used for azoxystrobin and fludioxonil sensitivity testing. Each isolate x fungicide

concentration combination was replicated twice with the whole trial repeated twice. From the mycelial growth measurements, the percentage inhibition was calculated, and statistical analyses done.

Statistical analysis

For azoxystrobin baseline and non-baseline isolates, percentage (%) inhibition over log concentration increased at a specific rate, and a power curve suitably fitted the trends in the data. For the fludioxonil baseline and non-baseline isolates, rate of percentage (%) inhibition over log concentration increased quickly, levelled out at intermediate concentrations, but again increased at higher concentrations. Thus, cubic polynomials fittingly described these trends in the data. Both functions were fitted over two petri dishes, representing each isolate concentration combination, separately for the two trials. EC_{50} and EC_{90} values were calculated employing the estimated parameters of the regression functions for each isolate at each trial. EC_{50} and EC_{90} values were subjected to analysis of variance (ANOVA) considering trials as block replicates for each isolate. Ward's Minimum Variance Clustering Method was used to cluster isolates in groups based on their inhibition patterns over fungicide concentrations. Isolate degrees of freedom in above-mentioned ANOVA's were then broken down into groups and isolates within groups to test differences between isolate groups. Means for significant effects were compared using Fisher's least significant difference. ANOVA was conducted using the GLM procedure of SAS statistical software (Version 9.4, SAS Institute Inc., Cary, NC, USA) and regression analysis using the NLIN procedure of SAS.

RESULTS

Efficacy of different azoxystrobin concentrations *in vitro* on mycelium growth

The sensitivity of a total of 51 *P. nicotianae* isolates (Table 1) unexposed to stobilurins were tested as well as 60 isolates (Table 1), potentially exposed to this fungicide group (non-baseline). In the pilot study, SHAM reduced mycelial growth significantly when used alone, of all 20 isolates in both populations, compared to the unamended control. Both populations were tested with SHAM present to block alternative respiration routes. In a pilot study it was seen that the addition of 100 µg/ml SHAM did significantly reduce the mycelial growth for all tested isolates in comparison to an unamended CMA control. After 7 days the unamended control plates of both populations had an average colony growth of 70.98 cm and the colonies that grew on the SHAM amended plates only 25.85 cm, which was significantly less. The EC_{50} shifted from ±100 µg/ml when only azoxystrobin was added and fell to 0.5 µg/ml when azoxystrobin and SHAM was used in combination in the pilot trials (data not shown). Thus,

with SHAM added, the pathway was effectively blocked, and the inhibition of mycelial growth could be tested accurately.

The initial Ward's cluster indicated four isolate sensitivity groups for azoxystrobin baseline isolates while the non-baseline population had five different sensitivity groups (Table 2). The ANOVA of the mean EC_{50} and EC_{90} values indicated significant (P<0.0001) differences between isolates [Group (IsolateNr)] within the different groups for both populations (baseline and non-baseline). This indicated that variation existed between isolates within groups with regards to their fungicide sensitivity. Between the sensitivity groups within each population, it was furthermore seen that based on abovementioned variables, there was also significant (P<0.0001) differences (Table 3 and 4).

Within the baseline population, group 1 had a mean EC_{50} value of 0.05 µg/ml and EC_{90} of 12.81 µg/ml (Table 2). Groups 3 and 4 had statistically similar EC_{50} values (0.18- and 0.19 µg/ml, respectively) that were significantly higher than the EC_{50} values of groups 1 and 2. Group 2 had the lowest mean EC_{50} value of 0.01 µg/ml that was significantly lower than the mean of group 1 that was 0.05 µg/ml (Table 2). In terms of EC_{90} values the differences between the groups was much more pronounced (Table 2). Group 4 had a mean EC_{90} of 83.96 µg/ml that was significantly more than the mean of group 3 (36.83 µg/ml). The mean EC_{90} of group 1 was 12.81 µg/ml, which was significantly lower than groups 3 and 4 and but higher than group 2. The mean EC_{90} of group 2 was again statistically the lowest at 4.28 µg/ml (Table 2).

The results of the population that were potentially exposed to the fungicides before were slightly different. The mean EC_{50} values of groups 1 and 5 were statistically similar (0.11- and 0.10 µg/ml, respectively). Despite being low, these were statistically higher than that of group 3 at 0.04 µg/ml. The mean EC_{50} value of group 2 was 0.30 µg/ml, which was significantly 2-3 times higher than the three groups mentioned above. The highest EC_{50} was that of group 4 at 0.46 µg/ml (Table 2). The mean EC_{90} data indicated that this value for group 3 was 11.45 µg/ml that was also statistically the lowest EC_{90} of all five groups. Group 1 had a significantly higher mean EC_{90} value (17.06 µg/ml) compared to group 3. The EC_{90} value for group 5 was third highest at 26.83 µg/ml that were followed by group 4 (52.59 µg/ml) and group 2 with the highest mean EC_{90} value of 84.85 µg/ml (Table 2).

Efficacy of different fludioxonil concentrations *in vitro* on mycelium growth

Ward's Clustering analysis was also done to indicate fludioxonil sensitivity groups within abovementioned two *P. nicotianae* populations. The clustering indicated that the isolates from both populations could be divided into five different fludioxonil sensitivity groups (Table 5). For both populations, the ANOVA of the EC_{50} and EC_{90} data displayed that there were significant (P < 0.0001) differences between groups and the isolates within the groups were also significantly (P < 0.0001) different (Table 6 and 7).

The EC_{50} values of the previously unexposed population (baseline) data showed that the statistically highest mean was seen for group 5 at 1613.52 µg/ml. The second highest mean EC_{50} was that of group 4 at 1111.80 µg/ml. The means for group 1-3 were statistically much lower and was respectively 5.56, 25.03 and 77.63 µg/ml (Table 5). In the case of the mean EC_{90} results, group 4 had the highest mean at 9929.30 µg/ml followed by a significantly lower mean for group 5 of 7718.50 µg/ml. The lowest mean EC_{90} value was recorded for group 3 (1988.50 µg/ml). The mean EC_{90} of groups 1 and 2 was 4907.60 µg/ml and 4021.80 µg/ml respectively that was statistically the third and fourth highest means (Table 5).

In the previously, possibly exposed population (non-baseline), group 5 had the significantly highest mean EC_{50} value of 84.79 µg/ml. The mean EC_{50} values of the other 4 groups were also statistically different from one another but were in value much lower than that of group 5. The respective group values of 1-4 was 5.87, 3.10, 8.93 and 9.95 µg/ml (Table 5). In terms of mean EC_{90} values in this population, the ranking of the groups also changed. Group 4 had the statistically highest mean of 6809.90 µg/ml, followed by groups 1 (5748.82 µg/ml) and 3 (5472.36 µg/ml). The mean of group 2 was 4987.18 µg/ml and group 5 had the statistical lowest mean of 1090.50 µg/ml (Table 5).

DISCUSSION

This study is the first to present mycelial growth of baseline and non-baseline populations *in vitro* of South African *P. nicotianae* isolates to azoxystrobin and fludioxonil, specifically for the control of postharvest brown rot on citrus. Mycelia is the main somatic structure which can survive in diseased material in consecutive growing seasons, if conditions are favourable (Gray *et al*., 2018). Thus, mycelial responses towards the respective fungicides were tested. Any chemical that can reduce the mycelial growth of a pathogen, has the ability to reduce the pathogen to cause disease (Matheron and Porchas, 2000; Errampalli, 2004). The current study established the mean baseline and non-baseline EC_{50} and EC_{90} values for a previously unexposed *P. nicotianae* population and a population possibly previously exposed to azoxystrobin and fludioxonil. Both fungicides are under investigation for postharvest use to control *P. nicotianae,* which causes brown rot on citrus fruits.

To effectively evaluate the sensitivity range of azoxystrobin, SHAM was added in the current study at 100 µg/ml, which was the suitable concentration to mix with the fungicide concentration range (Rebollar-Alviter *et al*., 2007), that was determined in a pilot trial. Activation of the alternative respiration pathway takes place when QoI are present, as these fungicides block the main respiration pathway and subsequently the pathogen can grow *in vitro*. *In vitro* studies testing strobilurin fungicides, with the use of the pathway inhibitor SHAM, has been used extensively (Kanetis *et al*., 2008).

The inhibitory effect of azoxystrobin towards *Phytophthora* has been studied previously and to a larger extend (Ma *et al*., 2018) compared to fludioxonil. In the current study, comparison of the EC_{50} and EC_{90} led to different sensitivity groups being identified within the populations based on Ward's clustering analyses. The differences in sensitivity to QoI *in vitro* could be as a result of their differences in genetics. For the QoI chemical class, the resistance of the pathogen is considered to be controlled by one mitochondrial gene and resistance risks are considered high (Fabritius *et al*., 1997; Ziogas *et al*., 2002). The mean EC_{50} values of azoxystrobin for *P. nicotianae* ranged for the different sensitivity groups within the baseline and non-baseline populations, between 0.01 and 0.46 µg/ml. The EC_{90} values for baseline and non-baseline populations of the different sensitivity groups ranged from 4.28 to 84.85 µg/ml. The sensitivity ranges of the baseline and non-baseline populations of EC_{50} (0.01 to 0.19 µg/ml as oppose to 0.04 to 0.46 µg/ml) and EC_{90} (4.28 to 83.96 µg/ml as oppose to 11.45 to 84.85 µg/ml) values were so similar that it could be argued that a sensitivity shift has not taken place between the populations.

In contrast to findings in this study, was a study conducted in 2000, where the efficacy of fluazinam, azoxystrobin, fosetyl-Al, metalaxyl and dimethomorph tested on three life stages (zoospore germination, growth and sporulation) of three *Phytophthora* spp. namely, *P. parasitica* (syn *P. nicotianae*), *P. capsici* and *P. citrophthora* (Matheron and Porchas, 2000). The EC_{50} of azoxystrobin on *P. parasitica*'s mycelial growth was indicated as >3000 µg/ml. The study suggested that the alternative respiration pathway was accessed when growing on nutrient rich agar which led to the low level of sensitivity to azoxystrobin. The EC_{50} value in that study (>3000 µg/ml) was much higher than what was found in the current pilot study (±100 µg/ml), where only azoxystrobin was added, and no SHAM. Another study where no SHAM was added and only azoxystrobin was tested against *P. nicotianae*, the EC_{50} values ranged between 56 to 165 µg/ml (Kuhajek *et al*., 2003), which was more similar to what was found in the current pilot study (±100 µg/ml), but was still significantly higher than the EC_{50} values in the current study that was amended with azoxystrobin and SHAM in combination (0.01 to 0.46 µg/ml).

Azoxystrobin, in combination with SHAM, was tested against *P. cactorum,* collected from strawberries' crown- and leather rot, where the EC_{50} values ranged from 0.10 to15 µg/ml (Rebollar-Alviter *et al*., 2007), which was again higher than the current study's *P. nicotianae* EC_{50} values. Another study found that azoxystrobin, also amended with SHAM, inhibited *P. capsici* mycelial growth with mean EC_{50} values of 14.40 to 21.04 µg/ml and ranged overall from 1.23 to 86.10 µg/ml (Qian *et al.,* 2006). This range was very similar to the current study's EC_{90} values (4.28 to 84.85 µg/ml), which could indicate that other *Phytophthora* spp. are less sensitive towards azoxystrobin than *P. nicotianae*. A recent study was conducted with benzothiostrobin, a strobilurin fungicide, in the presence of 100 µg/ml SHAM, again towards

P. capsici, which causes pepper blight. The EC_{50} values from different populations of the mycelial growth ranged from 0.95 to 2.46 µg/ml (Ma *et al*., 2018), which were relatively higher than this study's EC_{50} values.

Penicillium italicum and *P. digitatum* are two citrus pathogens and their mycelial growth was tested against azoxystrobin, with the presence of SHAM (Kanetis *et al*., 2008). Similar to the current study, the mean EC_{50} values for *P. italicum* was 0.029 µg/ml, for only 33 isolates. As for the other four isolates the mean EC_{50} was more than 0.772 µg/ml, which was regarded as a resistant population. *P. digitatum* had a mean EC_{50} value of 0.014 µg/ml (Kanetis *et al*., 2008). The EC_{50} values obtained in that study was very similar to the EC values of azoxystrobin in the current study. EC_{50} are usually calculated to assess whether the chemical in question can control the growth of a pathogen. Nevertheless, reducing the growth by 50% will usually not provide satisfactory disease management. Therefore, EC_{90} values could be more beneficial (Matheron and Porchas, 2000) and none of these studies provided the EC_{90} values. The EC_{90} values of azoxystrobin in the current study were still relatively low indicating a good level of effectivity in inhibiting the mycelial growth of *P. nicotianae*.

A very wide range of EC values was recorded for fludioxonil towards *P. nicotianae* in the current study. The EC_{50} baseline values ranged from 5.56 to1613.52 µg/ml and non-baseline 3.10 to 84.79 µg/ml. The EC_{90} baseline values ranged from 1988.5 to 9929 µg/ml. and non-baseline values 1090.50 to 6809.90 µg/ml. This could indicate that again no sensitivity shift occurred between baseline and non-baseline populations. The only study that could be found with fludioxonil and *P. nicotianae,* was a study conducted in 2018 where fludioxonil and metalaxyl-M were used in combination against mycelium growth of *P. nicotianae.* The EC_{50} and EC_{90} was 0.393 µg/ml and 10.170 µg/ml respectively (Altin *et al*., 2018), but it is important to note that *Phytophthora spp.* are sensitive toward metalaxyl and in combination with fludioxonil, was very effective. Both EC_{50} and EC_{90} values are significantly less than what was found in the current study.

Fludioxonil was also tested against citrus pathogens *P. digitatum* and *Lasiodiplodia theobromae* mycelial growth in Florida. The EC_{50} values was 0.020 ug/ml and 0.012 ug/ml respectively (Zhang, 2007). Another study where fludioxonil was used against two citrus pathogens, *P. italicum* and *P. digitatum* mycelial growth, resulted in respective mean EC_{50} values of 0.005 µg/ml and 0.163 µg/ml (Kanetis *et al*., 2008). Similar to the Altin and Zhang (2007) studies, the EC values was again significantly much lower than what was found in the current study.

The results of this *in vitro* study show that the maximum concentrations of azoxystrobin and fludioxonil tested, effectively inhibited mycelial growth of *P. nicotianae,* compared to the growth on unamended control plates. Azoxystrobin was very effective against *P. nicotianae.* It had EC_{50} values that was in the same range as those in other studies (Kanetis *et al*., 2008),

or lower EC_{50} values than other studies (Matheron and Porchas, 2000; Kuhajek *et al*., 2003; Qian *et al.,* 2006; Ma *et al*., 2018). Nabi *et al*. (2017) stated that fludioxonil is effective against a broad spectrum of fungi especially *Rhizoctonia*, *Botrytis*, *Aspergillus*, *Fusarium*, *Monilinia* and *Penicillium* spp. but not effective against oomycetes. This contrasts with what was found in the current study as fludioxonil did inhibit *P. nicotianae* mycelial growth, however the EC_{50} values were much higher than for other pathogens in other studies (Zhang, 2007; Kanetis *et al*., 2008; Altin *et al*., 2018).

With the establishment of a baseline sensitivity range of any pathogen towards a fungicide is important as it monitors for possible resistance and, to effectively manage it (Qu et al., 2016). Regarding the baseline and non-baseline sensitivity groups of both fungicides in the current study, it was seen that there was little difference in the EC values of the two populations. This indicated that currently there is little or no sensitivity shift between the populations of both fungicides in South Africa. The results obtained from this study furthermore shows that both fungicides were effective against *P. nicotianae in vitro*. These fungicides should therefore be tested *in vivo* to evaluate if it can effectively control brown rot as there is currently nothing registered in South Africa to control this disease at the postharvest stage.

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TABLES AND FIGURES

Table 1*: Phytophthora nicotianae* isolates representing previously unexposed and potentially exposed populations collected from various citrus growing regions in South Africa.

Isolate	Location	Province	Isolate	Location	Province
P10[1]	Stargrow	Western Cape	N7[2]	Clanwilliam	Western Cape
P22[1]	Namakwaland	Western Cape	N8[2]	Clanwilliam	Western Cape
P48[1]	Paksaam	Eastern Cape	N9[2]	Clanwilliam	Western Cape
P133[1]	Apapanzi	Eastern Cape	N10[2]	Clanwilliam	Western Cape
P201[1]	Mistkraal	Eastern Cape	N11[2]	Clanwilliam	Western Cape
1[1]	Nelspruit	Mpumalanga	N12[2]	Clanwilliam	Western Cape
2[1]	Nelspruit	Mpumalanga	N13[2]	Clanwilliam	Western Cape
3[1]	Nelspruit	Mpumalanga	N14[2]	Clanwilliam	Western Cape
4[1]	Nelspruit	Mpumalanga	N15[2]	Clanwilliam	Western Cape
5[1]	Nelspruit	Mpumalanga	N16[2]	Clanwilliam	Western Cape
6[1]	Nelspruit	Mpumalanga	N17[2]	Clanwilliam	Western Cape
7[1]	Nelspruit	Mpumalanga	N18[2]	Clanwilliam	Western Cape
8[1]	Nelspruit	Mpumalanga	N19[2]	Clanwilliam	Western Cape
9[1]	Nelspruit	Mpumalanga	N20[2]	Clanwilliam	Western Cape
10[1]	Nelspruit	Mpumalanga	N21[2]	Clanwilliam	Western Cape
11[1]	Nelspruit	Mpumalanga	N22[2]	Clanwilliam	Western Cape
12[1]	Nelspruit	Mpumalanga	N24[2]	Clanwilliam	Western Cape
13[1]	Nelspruit	Mpumalanga	N25[2]	Clanwilliam	Western Cape
14[1]	Nelspruit	Mpumalanga	N26[2]	Clanwilliam	Western Cape
15[1]	Nelspruit	Mpumalanga	N27[2]	Clanwilliam	Western Cape
16[1]	Nelspruit	Mpumalanga	N28[2]	Clanwilliam	Western Cape
17[1]	Nelspruit	Mpumalanga	N29[2]	Clanwilliam	Western Cape
18[1]	Nelspruit	Mpumalanga	N30[2]	Clanwilliam	Western Cape
19[1]	Nelspruit	Mpumalanga	N32[2]	Clanwilliam	Western Cape
20[1]	Nelspruit	Mpumalanga	N33[2]	Clanwilliam	Western Cape
21[1]	Nelspruit	Mpumalanga	N34[2]	Clanwilliam	Western Cape
22[1]	Nelspruit	Mpumalanga	N35[2]	Clanwilliam	Western Cape
23[1]	Nelspruit	Mpumalanga	N36[2]	Clanwilliam	Western Cape
24[1]	Nelspruit	Mpumalanga	N37[2]	Clanwilliam	Western Cape
25[1]	Nelspruit	Mpumalanga	N38[2]	Clanwilliam	Western Cape
26[1]	Nelspruit	Mpumalanga	N39[2]	Clanwilliam	Western Cape
27[1]	Nelspruit	Mpumalanga	N40[2]	Clanwilliam	Western Cape
28[1]	Nelspruit	Mpumalanga	M1[2]	Nelspruit	Mpumalanga
29[1]	Nelspruit	Mpumalanga	M2[2]	Nelspruit	Mpumalanga
30[1]	Nelspruit	Mpumalanga	M3[2]	Nelspruit	Mpumalanga
31[1]	Nelspruit	Mpumalanga	M4[2]	Nelspruit	Mpumalanga
32[1]	Nelspruit	Mpumalanga	M6[2]	Nelspruit	Mpumalanga
33[1]	Nelspruit	Mpumalanga	M7[2]	Nelspruit	Mpumalanga
34[1]	Nelspruit	Mpumalanga	M8[2]	Nelspruit	Mpumalanga
35[1]	Nelspruit	Mpumalanga	M9[2]	Nelspruit	Mpumalanga
36[1]	Nelspruit	Mpumalanga	M10[2]	Nelspruit	Mpumalanga
37[1]	Nelspruit	Mpumalanga	M11[2]	Nelspruit	Mpumalanga
38[1]	Nelspruit	Mpumalanga	B1[2]	Brits	North-West
39[1]	Nelspruit	Mpumalanga	B2[2]	Brits	North-West
40[1]	Nelspruit	Mpumalanga	B3[2]	Brits	North-West
41[1]	Nelspruit	Mpumalanga	B4[2]	Brits	North-West
42[1]	Nelspruit	Mpumalanga	B5[2]	Brits	North-West
44[1]	Nelspruit	Mpumalanga	B6[2]	Brits	North-West
45[1]	Nelspruit	Mpumalanga	B7[2]	Brits	North-West
46[1]	Nelspruit	Mpumalanga	B8[2]	Brits	North-West
N1[2]	Clanwilliam	Western Cape	B9[2]	Brits	North-West
N2[2]	Clanwilliam	Western Cape	B10[2]	Brits	North-West
N3[2]	Clanwilliam	Western Cape	B12[2]	Brits	North-West
N4[2]	Clanwilliam	Western Cape	B15[2]	Brits	North-West
N5[2]	Clanwilliam	Western Cape	B30[2]	Brits	North-West
N6[2]	Clanwilliam	Western Cape			

[1]Isolates used in baseline sensitivity study

[2]Isolates used in non-baseline sensitivity study

Table 2: Mean EC_{50}, and EC_{90} values of different azoxystrobin sensitivity groups within unexposed (baseline) and previously exposed (non-baseline) *P. nicotianae* isolates based on Ward's cluster analyses.

***Phytophthora nicotianae* population**	**Group**	**EC_{50} (µg/ml)**	**EC_{90} (µg/ml)**
Previously unexposed population (Baseline) N=51	1	0.05 b	12.81 y
	2	0.01 c	4.28 z
	3	0.18 a	36.83 x
	4	0.19 a	83.96 w
	LSD	0.013	2.486
Previously exposed population (Non-baseline) N=60	1	0.11 c	17.06 y
	2	0.30 b	84.85 v
	3	0.04 d	11.45 z
	4	0.46 a	52.59 w
	5	0.10 c	26.83 x
	LSD	0.021	2.448

[1]Means followed by the same letter are not significantly different.

Table 3: Analysis of variance (ANOVA) of mean EC_{50} and $-_{90}$ values of azoxystrobin baseline isolates of four groups identified based on Ward's cluster analyses.

Source	EC_{50}				EC_{90}			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Trial	1	0.002	0.002	0.0031	1	76.226	76.226	0.0040
Group	3	0.435	0.145	<.0001	3	30499.793	10166.597	<.0001
Group (Isolate Nr)	47	0.203	0.004	<.0001	47	30499.793	79.934	<.0001
Error	50	0.011	0.000		50	419.735	8.394	
Corrected total	101	0.653			101	34752.671		

Table 4: Analysis of variance (ANOVA) of mean EC_{50} and $-_{90}$ values of azoxystrobin non-baseline isolates of five groups identified based on Ward's cluster analyses.

Source	EC_{50}				EC_{90}			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Trial	1	0.000	0.000	0.905	1	25.746	25.746	0.093
Group	4	1.185	0.296	<.0001	4	35917.813	8979.453	<.0001
Group (Isolate Nr)	55	0.321	0.005	<.0001	55	4786.764	87.032	<.0001
Error	59	0.040	0.000		59	524.149	8.883	
Corrected total	119	1.547			119	41254.474		

Table 5: Mean EC_{50}, and $_{90}$ values of different fludioxonil sensitivity groups within unexposed (baseline) and previously exposed (non-baseline) *P. nicotianae* isolates based on Ward's cluster analyses.

***Phytophthora nicotianae* population**	**Group**	**EC_{50} (µg/ml)**	**EC_{90} (µg/ml)**
Previously unexposed population (Baseline) N=51	1	5.56 d	4907.60 x
	2	25.03 d	4021.80 y
	3	77.63 c	1988.50 z
	4	1111.80 b	9929.30 v
	5	1613.52 a	7718.50 w
	LSD	25.252	300.73
Previously unexposed population (Non-baseline) N=60	1	5.87 d	5748.82 w
	2	3.10 e	4987.18 y
	3	8.93 c	5472.36 x
	4	9.95 b	6809.90 v
	5	84.79 a	1090.50 z
	LSD	0.839	182.97

[1]Means followed by the same letter are not significantly different.

Table 6: Analysis of variance (ANOVA) of mean EC_{50} and $_{90}$ values of fludioxonil baseline isolates of five groups identified based on Ward's cluster analyses.

Source	EC_{50}				EC_{90}			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Trial	1	42.311	42.311	0.732	1	96299.3	96299.3	0.174
Group	4	7194015.531	1798503.883	<.0001	4	213931827.6	53482956.9	<.0001
Group (Isolate Nr)	46	19536.746	424.712	0.2746	46	94171711.0	2047211.1	<.0001
Error	50	17868.256	357.365		50	2534223.1	50684.5	
Corrected total	101	7231462.844			101	310734061.1		

Table 7: Analysis of variance (ANOVA) of mean EC_{50} and $_{90}$ values of fludioxonil non-baseline isolates of five groups identified based on Ward's cluster analyses.

Source	EC_{50}				EC_{90}			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Trial	1	4.646	4.646	0.069	1	145879.5	145879.5	0.138
Group	4	35623.695	8905.923	<.0001	4	155767675.7	38941918.9	<.0001
Group (Isolate Nr)	56	1315.318	23.487	<.0001	56	32266550.0	576188.4	<.0001
Error	60	81.537	1.358		60	3876092.8	64601.5	
Corrected total	121	37025.197			121	192056198.0		

CHAPTER 3

Evaluation of azoxystrobin, fludioxonil and potassium phosphite for the postharvest control of Phytophthora brown rot of citrus

ABSTRACT

Brown rot is a postharvest disease of citrus caused by *Phytophthora* spp. Fungicide management of brown rot in South Africa currently consists only of preharvest strategies and nothing is registered for postharvest management of this disease. The objectives of this study was to evaluate the curative and protective efficacy of azoxystrobin (1125 µg/ml), fludioxonil (598 µg/ml) and potassium phosphite (1500 ug/ml) as aqueous dip treatments for the postharvest management of Phytophthora brown rot on different citrus types (lemons, oranges and mandarins). Additionally, azoxystrobin (2500 µg/ml for all three fruit types) and fludioxonil (2300 µg/ml for lemons and 4600 µg/ml for oranges and mandarins) amended wax was evaluated for the prevention of spreading of brown rot (nesting) within cartons during transit. Results indicated that the three tested fungicides have good curative action, reducing brown rot incidence significantly when the fungicide was applied 12 hrs after inoculation. Applications done 24 hrs after inoculation also provided some curative action but not as effective as earlier applications. Azoxystrobin and potassium phosphite furthermore provided very good protection against infection if inoculations were done up to 48 hrs after application on all three fruit types but fludioxonil did not fare as well. Interestingly, the protective ability of all three fungicides was better the longer the fungicides remained on the fruit before inoculation. Trials aimed at prevention of nesting during transit indicated that only azoxystrobin amended wax significantly reduced brown rot from spreading to healthy fruit when in contact, compared to the control. The data obtained from this study can add additional value to the already registered postharvest azoxystrobin and fludioxonil fungicides and preharvest registered potassium phosphite.

INTRODUCTION

South Africa is the second largest exporter of citrus fruit globally (FFED, 2019) and there are several *Phytophthora* diseases that threaten this industry. These include foot and root rot, branch and trunk cankers, gummosis and brown rot of citrus fruit. Brown rot is considered a localized problem as the infecting propagules depend on water and air movement to spread from the soil onto fruit in the tree (Graham and Feichtenberger, 2015). Brown rot epidemics occur worldwide mostly in wet seasons when fruit are in the early to late maturing stage (Graham and Menge, 2000; Ramallo *et al*., 2019). *Phytophthora nicotianae, P. citrophthora*,

P. hibernalis and *P. syringae* are the main *Phytophthora* species occurring on citrus and all four pathogens can cause brown rot on citrus fruit (Adaskaveg *et al*., 2015; Ramallo *et al*., 2019). The importance of the prevalent species in a specific area is dependent on the climate of that specific area. *Phytophthora nicotianae* is more prevalent in warmer production areas whereas *P. citrophthora* is more prevalent in cooler production areas (Adaskaveg and Förster, 2014).

Phytophthora spp. can persists in soil as long term surviving chlamydospores or oospores, in decayed roots or as mycelium on rotted fruit (Adaskaveg *et al*., 2015; Ramallo *et al*., 2019). When all the environmental conditions are optimal, including temperature and wetness duration, sporangia develop from the above-mentioned structures within 18 hrs. With adequate free water, motile zoospores are released which in turn splash to the foliage and fruit through rain or irrigation. On the fruit surface these propagules encyst, followed by germination (Adaskaveg *et al*., 2015). The optimal conditions for infection of fruit is a constant wetness period of at least 3 hrs and temperatures from 14-23°C (Adaskaveg *et al*., 2015). Infected fruit can fall off, but if no symptoms occur in the orchard, the symptoms may only appear postharvest while in storage or transit. Symptoms include firm, brown and leathery lesions on the rind with a very distinguishing odour (Adaskaveg *et al*., 2015; Ramallo *et al*., 2019). Brown rot infections of fruit could lead to secondary infections by *Geotrichum* and *Penicillium* spp. which is other important postharvest pathogens (Adaskaveg *et al*., 2015). In some countries it can take up to 40 days for export fruit to reach the final consumers, which provides a long time for decay to develop (Ramallo *et al*., 2019).

Brown rot of citrus fruits can lead to economic repercussions and integrated control strategies are needed to manage this disease (Gray *et al*., 2018). Phytophthora brown rot control is therefore considered a continuous challenge (Ramallo *et al*., 2019). The management of brown rot depends mainly on cultural practices before harvest that include skirting of trees, proper irrigation and removal of ground level vegetation (Adaskaveg *et al*., 2015; Gray *et al*., 2018). It was also suggested not to harvest from the lower parts of the canopy or to wait until the infected fruit drop. Regardless of all the cultural practices, chemical control still makes out an important part of management to prevent brown rot from developing while in transit or at arrival in the export markets (Gray *et al*., 2018). However, this gets complicated with the lack of registered fungicides for management of this disease and the risk of resistance development against phenylamides and even more recently, phosphonates (Gray *et al*., 2018; Ramallo *et al*., 2019).

Although heavy fruit losses can occur due to brown rot in the orchard, there is an additional threat. When fruit with no visible symptoms are packed in contact with healthy, uninfected fruit in a carton, the latent infections can develop, which can lead to overall decay as the rot spreads. This occurrence is termed nesting. This can lead to secondary wound

pathogens infecting the fruit, increasing losses. This postharvest problem therefore originates from preharvest infections. Preharvest chemicals to control brown rot include phosphonates, copper and dithiocarbamate fungicides that are applied as fruit and foliar sprays to protect fruit against splash dispersed propagules (Gray *et al*., 2018).

Graham and Feichtenberger (2015) stated that other areas known for citrus production such as Brazil and Florida use phosphite salts as foliar sprays to manage this disease. However, the overuse of certain chemicals led to resistance towards some of these chemicals making it imperative to consider other fungicides for brown rot control (Ramallo *et al*., 2019). Postharvest fungicides should be specifically selected to be incorporated in the integrated management strategy (Qu *et al*., 2016) of any postharvest disease to effectively manage it. Postharvest treatments for Phytophthora brown rot control were registered recently in the United States (Adaskaveg *et al*., 2015). This treatment consists of potassium phosphite in combination with heat treatments.

Heat treatments is not a suitable option for brown rot control in South Africa, as this country needs to do cold sterilization for false codling moth control on export citrus destined for the EU. At present, South Africa has no postharvest treatments registered for control of this disease. The *in vitro* work from chapter 2 indicated that azoxystrobin and fludioxonil effectively inhibited the mycelial growth of *P. nicotianae in vitro*. As potassium phosphite was shown to be effective as heated solutions, it is unknown if this active could also be effective if used as an ambient temperature postharvest dip in South Africa, because there is always the possibility that fruit can be damaged during heat treatments and this could lead to fruit being more susceptible to pathogens (Erwin and Ribeiro, 1996). Thus, the objectives of this chapter were:

1. determine the curative and protective ability of azoxystrobin, fludioxonil and potassium phosphite postharvest aqueous dip treatments up to 48 hrs pre-and post-inoculation using *P. nicotianae* zoospores, and
2. determine the ability of azoxystrobin and fludioxonil amended wax to prevent the nesting effect during fruit transit or storage.

MATERIALS AND METHODS

Fungicides

The chemicals that were used for the postharvest dip treatments and amended wax applications were azoxystrobin [Obstructo, 25% active ingredient (a.i.), suspension concentrate, ICA International Chemicals, South Africa], fludioxonil (Teacher, 23% a.i, suspension concentrate, ICA International Chemicals, South Africa) and potassium phosphite (Fighter, 55,5% a.i. water-soluble, Rolfes Agri, South Africa).

Citrus fruits

The dip and amended wax application trials were repeated on three different citrus types. These were lemons, mandarins and navel oranges. After harvest, fruit were washed with chlorine (H2H, 150 µg/ml, South Africa) over rotating brushes, and dried at ambient temperature in a drying tunnel of a mini pack line. The fruits were dipped the following day for 1 min in 500 µg/ml imazalil (Imazacure, 50% a.i, emulsifiable concentrate, ICA International Chemicals, South Africa), to prevent decay of fruit due to *Penicillium* spp. The fruit were dried at ambient temperature and stored for no longer than a week at 7°C before use. Fruit were moved from cold storage to ambient temperature 24 hrs before the trials began.

Isolates and zoospore production

A mixture of three *P. nicotianae* isolates were used as inoculum during the trials. These isolates were selected based on pilot zoospore production trials. Zoospore production was optimized from the Londsdale *et al.* (1988) method. Isolates were hyphal tipped from PARPH (Kannwischer and Mitchell, 1978) and grown on CMA (Sigma-Aldrich, St. Louis, Missouri, USA) media for 4-7 days in a dark room at 28°C. A damp, 80 mm diameter circular, autoclaved miracloth was placed on a 90 mm pea agar (Chen and Zentmyer, 1970) plate and inoculated with 10 *P. nicotianae* colonized CMA plugs. The inoculated pea agar plates were subsequently incubated at 28°C for 4 days in the dark. After the incubated period, the miracloth was transferred to a sterile 250 ml Erlenmeyer flask containing 1/7 diluted pea broth (Chen and Zentmyer, 1970).

These flasks were shaken for 48 hrs in an orbital shaker (Labcon, Maraisburg, South Africa) at 22°C and 160 rpm (revolutions per minute) in the dark. After the shake incubation period, the pea broth was poured off and 75 ml salt solution, prepared according to Chen and Zentmyer (1970), was added and shaken for 30 min at 160 rpm in the shake incubator. The salt wash step was repeated twice. After the last wash, 20 ml of the salt solution was poured into the flask. The flask was placed in the shake incubator at 22°C at 160 rpm, in the dark, for 24 hrs. After the incubated time, the salt solution was poured off and the miracloth was rinsed twice with 100 ml sterile distilled water and 40 ml of fresh distilled water was added. The flasks were incubated for 90 min to 3 hrs at 18°C for zoospores release. The zoospores were filtered through sterile miracloth into a falcon tube. Spores were kept at 19°C while quantifying, with the use of a haemocytometer. A zoospore concentration of 3×10^5 were used for each of the fruit inoculations. Before each inoculation, 100 µl was plated out on CMA plates and incubated at 29°C for 48 hrs. Plates were inspected and germination percentage was calculated from 50 observed zoospores. This was done to ensure zoospores and therefore inoculum was viable.

Fruit inoculation

Fruit used for the curative trials were wounded at two points on the fruit, an equal distance from the stem end. Wounding was done by using 60 grit autoclaved sandpaper (1 cm^2) to make superficial wounds by scraping the fruit flavedo, not going into the albedo. This wounding was aimed at simulating what happened in the field when fruit scrapes on the soil surface. The wounds were inoculated by placing a sterilized miracloth (1 cm^2), dipped in the prepared zoospore suspension, on the wound. Once the miracloth was dry, it was removed. Fruit were dip treated with the fungicides 6, 12, 24 or 48 hrs after inoculation. Fruit used for the protective trials were first wounded as described above and then treated with the respective fungicides, left to dry, and inoculated 6, 12, 24 and 48 hrs after treatment.

Curative and protective ability of azoxystrobin, fludioxonil and potassium phosphite as aqueous dips

The curative and protective ability of azoxystrobin, fludioxonil and potassium phosphite were tested for each fungicide at one concentration only. All trials were separately repeated on three different citrus fruit types namely lemons, oranges and mandarins. There were three replicates of 24 fruit for each fungicide x time point combination. The dosage for azoxystrobin was 1125 µg/ml, for fludioxonil 598 µg/ml (both according to label recommendations) and for potassium phosphite it was 1500 ug/ml (Adaskaveg *et al.*, 2015). A 25 L aqueous solution was prepared for each fungicide in municipal water at ambient temperature. During the trials, the solutions were stirred continuously.

For the azoxystrobin and fludioxonil trials, fruit was dipped in the fungicide solutions for 60 s and for the potassium phosphite, fruit was dipped for 15 s. Wounded, un-inoculated negative controls consisted of fruit only dipped in the respective fungicides at the dosages mentioned above and evaluated after 4-5 days. In order to evaluate possible phytotoxicity of potassium phosphite on mandarin fruit, a double dosage (3000 ug/ml) dip treatment as negative control was added. After the double dosage treatment, the fruits were stored for one month at 7°C, followed by one week at ambient temperature. After the week at ambient temperature, fruit was inspected for phytotoxic damage.

Wounded, inoculated positive control fruit were dipped in water only. After the respective different dip treatments, fruit were left to dry at ambient temperature in the laboratory. All dried fruit were placed in plastic crates and covered with polyethylene bags. Wet paper towel balls were also placed inside the crates to ensure high relative humidity conditions during incubation. After closing the bags with sticky tape, the enclosed trays with fruit were incubated in the dark at 28°C. The fruit was evaluated for brown rot incidence 4-5 days after inoculation.

Each fruit was rated based on the development of brown rot at the two inoculation points. Based on the number of infected wounds on a fruit, each fruit got a rating of 0, 1 or 2.

Protective ability of azoxystrobin and fludioxonil amended wax applications

Fruit were wounded in the same manner as described above. After wounding, fruit was treated with wax amended with the respective fungicides on a custom-built pack line resembling a line in a commercial packhouse. All fruit types were treated with wax (Endura-Fresh QDP 18, JBT Corporation, Cape Town, South Africa) amended with 250 ml/ 25 L (2500 µg/ml) of azoxystrobin. For fludioxonil, lemons were treated with wax amended with 250 ml/ 25 L (2300 µg/ml) of fungicide and the oranges and mandarins were treated with wax amended with 500 ml/ 25 L (4600 µg/ml) of fungicide. The dosages of the different fungicides used on the different fruit types were according to the fungicide label or recommendations of the registration holder. The amended wax was applied at a rate of 1.2 L ton^{-1} of fruit. After applying the amended wax coating, fruit were dried in the drying tunnel and at ambient temperature in the laboratory.

One-week-old inoculated fruit displaying characteristic brown rot symptoms were packed in crates and surrounded by healthy fruit treated with the fungicide amended wax. Each fungicide amended wax treatment, as well as the wounded, untreated control treatment, was replicated four times with each replicate consisting of 20 fruit. When packing the treated and untreated fruit in the crates, it was ensured that the treated or untreated wounds were in contact with the brown rot symptomatic fruit. Four treated fruit surrounded one symptomatic fruit and three symptomatic fruit were placed in a carton. Moisture balls were again added to ensure high humidity during incubation. Fruit were enclosed with plastic bags and incubated at 28°C for 7 days in the dark. After incubation, the number of newly infected fruit were determined based on whether the wound showed brown rot infestation or not.

Statistical analyses

Percentage (%) brown rot was calculated by expressing the number of wounds (curative and protective) or fruit (nesting) with brown rot as a percentage of the total number of wounds or fruit per box. Percentage brown was subjected to analysis of variance (ANOVA) using the GLM procedure of SAS statistical software (Version 9.4, SAS Institute Inc., Cary, NC, USA). Shapiro-Wilk test was performed on the standardized residuals from the model to test for deviation from normality (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% confidence level to compare treatment means (Ott, 1998). A probability level of 5% was considered significant for all significance tests.

RESULTS

Curative and protective ability of azoxystrobin, fludioxonil and potassium phosphite as aqueous dips

Curative

Analysis of variance (ANOVA) of percentage brown rot data obtained from curative action trials done on lemons, oranges and mandarins indicated that there was a significant ($P < 0.0001$) treatment fungicide x time interaction for all fruit types (Table 1). The curative results from lemons indicated that if fruit was treated with azoxystrobin 6 hrs after *P. nicotianae* zoospore inoculation, no brown rot developed. When treated 12 hrs after inoculation, the mean percentage brown rot increased to 5.6% that was statistically the same as at 6 hrs (Figure 1A). However, when treatment occurred 24 hrs after inoculation, the percentage brown rot increased significantly to 37.5%. With treatment 48 hrs after inoculation, the mean further increased significantly to 95.8%. This was significantly more than even the untreated control (76.0% brown rot), indicating the loss of the curative action of azoxystrobin (Figure 1A).

The same trend was seen for the fludioxonil curative treatment. Treatment 6 hrs after inoculation again resulted in the lowest percentage brown rot (1.4%) that was statistically similar to the mean observed at 12 hrs (2.8%) (Figure 1A). The percentage brown rot increased significantly to 25.0% with treatment at 24 hrs. However, all these time points had statistically lower means than was observed in the untreated, inoculated control. Fludioxonil treatment 48 hrs after inoculation resulted in 72.2% brown rot that was statistically the same as the mean of the untreated, inoculated control (80.2% brown rot) (Figure 1A). A slightly different trend was seen for the potassium phosphite curative treatment on lemons. Treatment 6 and 12 hrs after inoculation led to a mean percentage brown rot that was statistically the same (26.4%). When treated 24 hrs after inoculation, the mean percentage brown rot increased to 68.1%, which was statistically higher than the means observed at 6 and 12 hrs (Figure 1A). This was a statistically lower mean than when treatment occurred 48 hrs (91.7%) after inoculation, but was statistically similar to the inoculated, untreated control.

Brown rot control results obtained from the curative treatment trials on oranges indicated that fruit treated with azoxystrobin 6 hrs after inoculation, had a mean percentage brown rot of 6.9%, which was statistically similar to the mean observed from fruit treated 12 hrs after inoculation (1.4%) (Figure 2A). The percentage brown rot increased significantly to 37.5% with treatment at 24 hrs after inoculation. However, 48 hrs (52.8%) was statistically the same as the inoculated untreated control (61.5%) (Figure 2A). A similar trend was observed for the curative results obtained with fludioxonil on oranges. Treatment 6 hrs after inoculation had the third highest mean percentage brown rot of 20.8%. The percentage brown rot decreased

significantly to 0.0% with treatment at 12 hrs. When treatment occurred 24 hrs after inoculation, the percentage brown rot increased statistically to 55.6% that was statistically similar to the control. Again, treatment 48 hrs after inoculation, resulted in no control and the mean percentage brown rot that developed was 72.2% which was significantly more than the inoculated untreated control (61.5%) (Figure 2A).

The potassium phosphite treatment on oranges gave overall very good curative control up to 48 hrs after inoculation and had a slightly different trend than for azoxystrobin and fludioxonil. Mean percentage brown rot observed for 6 and 12 hrs was 1.4% and 2.8% respectively, which was statistically the same. However, the mean percentage brown rot decreased with treatment at 24 hrs after inoculation and was 0.0%, statistical similar to the fludioxonil treatment 12 hrs after inoculation. The mean percentage brown rot increased to 15.3% 48 hrs after inoculation. However, this was still significantly lower than the inoculated untreated control (Figure 2A).

The percentage brown rot data from the curative trial on mandarin fruit showed a similar trend as that observed on the oranges. Data indicated that if fruit was treated with azoxystrobin 6 hrs after inoculation the mean percentage brown rot was 22.2%. When treated 12 hrs after inoculation, the mean percentage decreased to 15.3% that was statistically the same as the hour 6 treatment. However, when treatment occurred 24 hrs after inoculation, the percentage brown rot increased significantly to 48.6%. With treatment 48 hrs after inoculation, the mean percentage brown rot increased again significantly to 58.3%. However, the mean percentage brown rot observed at all four curative treatment time points was statistically lower than the inoculated untreated control, which had a mean of 76.0% (Figure 3A).

Fludioxonil treatment had the same trend as observed for azoxystrobin. The highest mean percentage brown rot (43.1%) were seen when treatment was done 6 hrs after inoculation. This was statistically more than the mean percentage brown rot observed at hour 12 treatment point (16.7%) (Figure 3A). The mean brown rot increased again significantly to 47.2%, statistically similar level to that observed with treatment at 6hrs after inoculation. The mean percentage brown rot for 48 hrs after inoculation (48.6%) was statistically similar to hrs 6 and 24, but again all treatment time points had mean percentages brown rot significantly lower than the control (Figure 3A). Potassium phosphite curative treatment results on mandarins were again slightly different. Hours 6, 12 and 24 were all statistically similar with means of respectively 4.2%, 5.6% and 13.9%. However, potassium phosphite treatment 48 hrs after inoculation of the mandarins resulted in a mean of 62.5% that was statistically the same as the inoculated, untreated control (Figure 3A).

Protective

The ANOVA of percentage brown rot resulting from protective ability trials on lemons, oranges and mandarins indicated again a significant ($P < 0.0001$) fungicide x treatment time interaction (Table 2). In the case of azoxystrobin, it was seen that when lemon fruit was inoculated 6, 12, 24 or 48 hrs after fungicide treatment, the mean percentages brown rot developing was statistically the same. The means ranged from 2.8% at 48 hrs to 15.3% at 12 hrs (Figure 1B). These means were all significantly lower than the untreated, inoculated control with a mean of 80.2%. Interestingly, the means declined with increasing time of inoculation after treatment (Figure 1B). Fludioxonil treatment on lemons had a similar trend but showed over all poorer protective ability in comparison to azoxystrobin and potassium phosphite. Inoculation 6 hrs after treatment resulted in 62.2% brown rot development, which was statistically lower than the control (80.2%). From 6 hrs after treatment to 12 hrs after treatment, it increased to 70.8%, which was statistically similar to the inoculated, untreated control (80.2%) (Figure 1B). When inoculation was done 24 hrs after treatment, the mean percentage brown rot decreased to 54.2% that was statistically lower than the control. The statistically best protective action with fludioxonil was seen when inoculation was done 48 hrs after treatment. For this inoculation time point, the mean was 30.6% (Figure 1B). It was therefore seen for fludioxonil, that the protective ability improved with increasing time of inoculation after treatment.

The protective ability of potassium phosphite on lemons had a different trend than for azoxystrobin and fludioxonil but the trend to protect the fruit was the same as there where better protection with increased time after the fungicide treatment. Mean percentage brown rot with inoculation 6 hrs after treatment was 44.4% and decreased significantly when inoculation occurred 12 hrs after treatment as the mean brown rot percentage decline to 33.3% (Figure 1B). The percentage brown rot decreased even further at the inoculation time points of 24 (12.5%) and 48 hrs (8.3%) after treatment that were statistically similar to each other. All the inoculation time points of protective potassium phosphite treatments had means that were statistically lower than the inoculated untreated control (80.2%) (Figure 1B).

Analysis of data for protective ability on oranges, indicated that if fruit was inoculated with *P. nicotianae* zoospores 6 and 12 hrs after azoxystrobin treatment, the mean percentage brown rot was statistically the same and respectively 19.4% and 13.9% (Figure 2B). However, when fruit was inoculated 24 and 48 hrs after treatment, no brown rot occurred (0.0%). This trend is similar to the lemons, as protective ability got better when inoculation was done with increasing time after fungicide treatment (Figure 2B). However, with potassium phosphite, at all four time points (6-48 hrs), no brown rot was observed (0.0%), which indicated an excellent protective ability on oranges. For both azoxystrobin and potassium phosphite treatment, all

inoculation time points had means that were statistically lower than the inoculated, untreated control (80.2%) (Figure 2B).

The percentage brown rot observed on oranges treated with fludioxonil was much higher at all time points than oranges treated with azoxystrobin and potassium phosphite. When inoculation occurred 6 (66.7%) and 12 hrs (75.0%) after treatment, it was statistically the same to the inoculated untreated control (80.2%) (Figure 2B). Again, the percentage brown rot decreased significantly to 31.9% with inoculation at 24 hrs. Inoculation 48 hrs after fludioxonil treatment resulted in 4.2% brown rot, which was again a significant decrease. Mean percentages brown rot observed 24 and 48 hrs after treatment were statistically lower than the control (Figure 2B). Again, better protective action was observed the longer the fungicide was present on the fruit before inoculation.

The mean percentage brown rot observed following protective trials on mandarins, were very low for all three fungicides. Treatment with azoxystrobin, showed that when fruit was inoculated 6, 12, 24 or 48 hrs after fungicide treatment, the mean percentages brown rot that developed was statistically the same, which was the same as the protective lemon data. Again, inoculation 6 hrs after treatment had mean percentage brown rot of 6.9%, hour 12 and 48 where both 2.8% and fruit inoculated 24 hrs after treatment had no brown rot lesions (0.0%). Means were furthermore all significantly lower than the untreated, inoculated control (77.1%) (Figure 3B).

Protective fludioxonil treatment on mandarins had a different trend than azoxystrobin. Mean percentage brown rot was 43.1% when inoculation was done 6 hrs after treatment. Inoculation at hour 12 resulted in statistically lower mean brown rot (25.0%) than inoculation at hour 6. In the case of inoculation 24 hrs after treatment, the mean percentage brown rot increased again to 40.3%, which was statistically similar to the mean of the 6 hrs inoculation time. However, 48 hrs after fludioxonil treatment, the brown rot percentage decreased significantly to 2.3%, which has the same trend as the other fruit for fludioxonil where hour 48 provided good protective action. Potassium phosphite protective data on mandarins had a statistical similar trend as for azoxystrobin where 6 hrs after treatment, the mean percentage brown rot was 6.9% and hrs 12-48, 2.8%, and all four time points were statistically the same (Figure 3B). Additionally, the mandarins showed no phytotoxic damage even with a double dosage of potassium phosphite (3000 µg/ml).

Protective ability of azoxystrobin and fludioxonil amended wax applications

The ANOVA of the percentage brown rot data resulting from nesting prevention trials on lemons, oranges and mandarins indicated that there was a significant fungicide (treatment) effect of respectively 0.0137, 0.0018, on lemons and oranges. However, on mandarins the fungicide effect was not significant (P = 0.5320) (Table 3). Thus, not one of the two tested

fungicides were effective on mandarins. In the case of lemons, analysis of the percentage brown rot data for wax amended with azoxystrobin and placed adjacent to rotted fruit, indicated that the mean percentage brown rot that developed was 41.7%, which was significantly lower than the mean observed on fruit from the wax only control (79.2%). The mean percentage brown rot that developed when fruit was covered with fludioxonil amended wax was 70.8% that was statistically similar to the mean of the control (Table 4).

With the oranges, there was a similar trend than with the lemons, as the mean percentage brown rot of the fruit treated with azoxystrobin amended wax was 56.3%, which was significantly lower than the unamended control mean with 100%. In the case of fruit covered with wax amended with fludioxonil, the percentage brown rot was 89.6% that was statistically the same as the unamended control. The percentage brown rot that developed on mandarins with treatment of azoxystrobin, fludioxonil and the control was respectively 91.7%, 95.8% and 97.9% (Table 4). It was seen that when fruit was covered with wax amended with azoxystrobin and fludioxonil, the mean percentage brown rot that developed were statistically the same as that of the wax only treatment and thus gave no protection.

DISCUSSION

Phytophthora pose continuous universal and economical challenges in all the citrus production areas and therefore integrated control strategies are needed to manage this pathogen effectively (Adaskaveg *et al*., 2015). Previous studies have emphasised the importance of alternative treatments for postharvest brown rot. This study constitutes the first evaluation of these fungicides in South Africa for the control of postharvest Phytophthora brown rot. Results obtained gave interesting insights into the efficacy of azoxystrobin, fludioxonil and potassium phosphite. These fungicides were tested in aqueous solutions for their curative and protective ability in controlling postharvest Phytophthora brown rot. Azoxystrobin and fludioxonil amended wax were furthermore evaluated for its ability to prevent the nesting effect, or spreading of brown rot, occurring within export cartons.

Zoospores were chosen as the source of inoculum, as opposed to using mycelial plugs, as it represents more accurately what happens in the field. In orchards, zoospores are splashed upward from the soil to the fruit during raining periods or irrigation. Once on the fruit, the zoospores encyst, germinate and cause characteristic brown rot symptoms (Adaskaveg *et al*., 2015). In some cases, the mean percentage brown rot of the untreated control was lower as other time points (curative action on oranges). This could be the result of temperatures fluctuated in certain areas within the incubation room. Nonetheless, the means were still high enough to make clear conclusions regarding the efficacy of the different fungicides.

The three fungicides were tested at four different time points and at specific dosages for each. These dosages were based on the registered label recommendations as well as previous studies. Results from all three fruit types indicated that the three fungicides had very good curative action if treatment was done 12 hrs after *P. nicotianae* zoospore inoculation. The effectiveness of the three tested fungicides' curative action in the current study is very noteworthy, as germination of *Phytophthora* zoospores can already occur as little as 3 hrs after infection (Adaskaveg *et al*., 2015). Similar curative results were seen when azoxystrobin and fludioxonil were tested against the postharvest citrus pathogen *P. digitatum* when treatment occurred 9 to 21 hrs after inoculation (Kanetis *et al*., 2007). Efficacy was also good at early timings but decreased when time increased between inoculation and treatment.

In the current study, treatment 24 hrs after inoculation resulted in a significant reduction in brown rot development, except for lemons treated with potassium phosphite and oranges treated with fludioxonil. Interestingly, only potassium phosphite had excellent curative ability on oranges when fruit were inoculated 48 hrs after treatment, but not on mandarins, where azoxystrobin and fludioxonil exhibited better curative action. When citrus fruit are infected in the orchard, it is usually during the raining season and fruits are not picked soon after a rain shower, as the fruit rind can get damaged. Thus, the period between infection in the orchard and the postharvest treatment in the packhouse, can be longer than 24 hrs. However, temperatures in the winter are usually much lower than *Phytophthora*'s optimal growth temperature. This can slow down the infection process that can make curative treatments successful (Adaskaveg *et al*., 2015).

Adaskaveg and Förster (2014) tested both potassium phosphite and azoxystrobin as curative and protective treatments. They concluded that only potassium phosphite displayed good curative action, which contrasts with what was found in the current study, as azoxystrobin in this study was very effective on all fruit types both curatively and protectively. Azoxystrobin (600 µg/ml) was tested on oranges 12 hrs after *P. citrophthora* zoospore inoculation and resulted in 81.2% brown rot incidence (Adaskaveg and Förster., 2014). In contrast, the current study resulted in azoxystrobin curative action on oranges at hour 12, resulted in only 1.4% brown rot development, which was significantly lower than observed in the Adaskaveg and Förster (2014) study. However, it is important to note that the active ingredient dosage used in the current study was double of the abovementioned 2014 study.

The curative efficacy of potassium phosphite on orange fruit inoculated with *P. citrophthora* zoospores 12 hrs before treatment at a dosage of 1260 µg/ml, resulted in 5% brown rot (Adaskaveg and Förster., 2014). However, in the current study with the treatment of potassium phosphite on oranges 12 hrs after inoculation resulted in 2.8% brown rot. This was similar to the results obtained by Adaskaveg and Förster (2014). The 2015 curative study on oranges from Adaskaveg *et al*. gave a 96% brown rot control when potassium phosphite (1500

µg/ml) was tested as dip treatments of 15 s, 18 hrs after inoculation with *P. citrophthora* zoospores. This was again similar to what was found in the current study. When treatment occurred 24 and 30 hrs after inoculation, brown rot incidence was 7.7% and 17.2% respectively (Adaskaveg *et al*., 2015). In the current study, oranges inoculated 24 hrs before treatment, resulted in no brown rot while inoculation 48 hrs before treatment resulted in 15.3% brown rot, which was similar as the Adaskaveg *et al* (2015) study.

Ramallo *et al* (2019) also did a study on lemons testing different potassium phosphite formulations at 2000 µg/ml. In these trials, lemons were inoculated with *P. citrophthora* mycelial plugs 24 hrs before treatment. This resulted in brown rot reductions of 20-40% compared to the untreated control. Brown rot incidence in the current study where lemons were inoculated 24 hrs before treatment resulted in 68.1% brown rot reduction compared to the inoculated untreated control of 76%, which is basically only a 10% reduction and thus lower efficacy to what Ramallo *et al* (2019) found.

Both azoxystrobin and fludioxonil (2000 µg/ml) sensitivity was tested curatively against other citrus pathogens, *Lasiodiplodia theobromae* and *Diaporthe citri*, causing postharvest stem end rot, as aqueous curative dips on lemons 24 hrs after inoculation, but were found to be ineffective as both resulted in 80% rot (Cerioni *et al*., 2017). This contrasted with what was found in the current study, as both azoxystrobin and fludioxonil resulted in good curative action when lemons were treated up to 24 hrs after inoculations.

The curative actions in the current study was very effective up to a certain time point but the protective action of the tested fungicides was more effective. Azoxystrobin and potassium phosphite had very good protective action on all three fruit types as these two fungicides reduced brown rot development significantly (0-44%), from the inoculated untreated control. On azoxystrobin treated fruit, brown rot incidence did not go above 20% with inoculation up to 48 hrs after treatment. Adaskaveg and Förster (2014) inoculated the fruit 12 hrs after azoxystrobin treatment (600 µg/ml) and this resulted in 10.5% brown rot developing, which was highly effective. Similarly, in the current study, the percentage brown rot on oranges, when inoculation occurred 12 hrs after azoxystrobin treatment, was just slightly higher at 13.9% which is also still effective.

Interestingly, in the current study, inoculations up to 48 hrs after treatment with potassium phosphite resulted in no decay, which could be due to initial contact action of the fungicide which provides protection and thereafter, possibly, local systemic activity that protect the fruit from established infection to develop further (Adaskaveg and Förster, 2015). When Adaskaveg and Förster (2014) inoculated orange fruit 12 hrs after potassium phosphite treatment, the resulting percentage brown rot was 1.3%. However, in the current study, potassium phosphite treated fruit had no brown rot incidence when inoculation took place 12 hrs after treatment. Ramallo *et al* (2019) did a study testing different potassium phosphite

formulations at 2000 µg/ml on lemons using *P. citrophthora* mycelial plugs. Protective action was tested where inoculation took place immediately after treatment that resulted in limited control and, 7 days after treatment resulted in 50-60% less brown rot development. This is interesting as the fungicide seems to work more effectively the longer it is on the fruit, as can be seen from results in the current study.

Fludioxonil did not show the same protective ability as azoxystrobin and potassium phosphite but fludioxonil had low percentage brown rot incidence with all three fruit types if inoculation occurred 48 hrs after treatment. A trend that was seen for the protective action on all three fruit types, was that the longer the fungicide remained on the fruit, the better the action was. This could be due to a rind response, triggered by the treatment of the fruit, such as cell wall changes that leads to mechanical barriers or protective substances being formed (Ramallo *et al*., 2019). Additionally, Ramallo *et al* (2019) also tested treatments specifically aimed to reduce postharvest Phytophthora brown rot with fungicides ametoctradin and dimethomorph in combination, at two concentrations (250 and 500 µg/ml) protectively (inoculations that occurred immediately, 7, and 14 days after fungicide treatments) and curatively (treatments occurred 24 hrs after inoculation) with the use of *P. citrophthora* mycelial plugs. Their study concluded that the protective action was very good as it reduced brown rot incidence with 60 and 90% for the respective concentrations, compared to the control. The 7 and 14 days after treatment (protective activity) and the overall curative activity lacked.

In previous studies, it was indicated that dips longer than 15 s for potassium phosphite was not recommended because it was deemed to be impractical in the main season when large amounts of fruit are going through the packhouse and time is of the essence (Adaskaveg *et al*., 2015). Longer exposure times were furthermore regarded as risky due to the possibility of phytotoxicity that potassium phosphite may cause on the fruit. It is important to note that, with the specific dosage of potassium phosphite used in the current study (1500 µg/ml), and even with a double dosage of the active (3000 µg/ml), followed by cold storage for a month, and one week at ambient temperature, no phytotoxic damage of fruit was observed. The low percentage brown rot that occurred in the current study is an indication of how the single dosage was sufficient.

Previous postharvest studies mentioned had different results compared to the current study. This could be due to several reasons. For one, some of the other studies focussed on other *Phytophthora* spp. such as *P. citrophthora,* which can have different sensitivities to the tested fungicides. Inoculation methods in other studies were also different as that used in the current study. For example, zoospore drenched miracloth squares with wounding was used as inoculation technique as opposed to mycelial plugs, with and without wounds used in other studies. Pathogenicity of the isolates used to prepare the zoospore inoculum could also play an important role. Due to the possible variation in virulence between isolates, three isolates

were used to produce the zoospore inoculum mixture used in the current study. The last factor that could have led to different results obtained is the different citrus fruit types that were used (lemons, oranges and mandarins).

Different trends were observed on the different fruit types (Figs. 1-3), which could also be due to the susceptibility of the fruit rind, rind thickness or natural defence mechanisms that the fruit rind activates when infection of pathogens occur. The presence of antifungal constituents from citrus fruit has been shown to play a role in natural resistance (Ben-Yehoshua *et al*.,1992). In citrus, early maturing varieties (e.g. mandarins) have lower wax levels than later maturing varieties, as higher temperatures tend to stimulate wax production (Petracek., 1997.) Mandarins also presents less firmness and a softer texture and elasticity than oranges and lemons (Petracek., 1997; Nunes., 2008).

It is important to note that oranges and mandarins are usually treated with fungicides only once after harvest and marketed soon thereafter (Kanetis and Adaskaveg, 2007). Lemons can, however, be marketed months after storage and are therefore treated with postharvest fungicides before storage and again before leaving the storage facility (Kanetis and Adaskaveg, 2007). It is also important to note that lemons lose their natural antifungal activity after a long time of storage and thus a decrease in defence mechanisms (Ben-Yehoshua *et al*.,1992). It is therefore very important that the fungicide that is applied to the fruit, has extended protective abilities. The possibility therefore exists to treat the fruit initially with only azoxystrobin or potassium phosphite and later fludioxonil, each time with a different active, therefore avoiding resistance build up. Resistance build up towards a fungicide can be a serious problem because it will no longer be effective in management of diseases.

Another possible reason for different trends seen on the different fruit types in the current study is that each fruit has natural defence mechanisms. The current understanding of citrus fruits' natural biochemical and molecular resistance towards pathogens are still relatively unknown (Porat *et al*., 2001; Ballester *et al*., 2010). Although, it is known that citrus rinds do contain antifungal agents to contribute to its natural defence mechanisms and a citrus peel contain large amounts of volatile oils, coumarins and flavonoids which has the potential to withstand postharvest fungi (Chen *et al*., 2019). However, different citrus types produce different natural defence mechanisms responding to infection of pathogens (Ben-Yehoshua *et al*.,1992).

Fungicide applications are also done in fruit wax coating to improve appearance of fruit for marketing purposes or to prevent the loss of moisture (Kanetis and Adaskaveg, 2007). As postharvest *Phytophthora* can spread from brown rot infected fruit to healthy fruit during postharvest stages, such as de-greening, storage and/or transit (Adaskaveg *et al.*, 2015), should these treatments also be able to prevent this trasfer. In the current study, it was demonstrated that when azoxystrobin was incorporated in wax and the fruit was coated with

it and placed adjacent to one week old brown rot fruit, it gave significant protection from brown rot spreading from infected to healthy fruit, when compared to the control. However, this was only observed in lemons and oranges but not when mandarins were treated with amended wax. Although fludioxonil amended wax treatments did provide a reduction in the spread of infection between infected and healthy fruit, the reduction was not significant when compared to the control.

Based on the results of the current study, the protective action of the three tested fungicides was better than the curative action. This agrees with what Nanni *et al* (2016) stated that when the active ingredient is already present on the fruit, it will provide better protection when fungal propagules land on the surface as it will interrupt the fungal development (protective). However, the management of diseases gets more complicated when the fungus is already present on the fruit and then treated (curative).

Azoxystrobin and fludioxonil are already registered for the control of *Penicillium* spp. in South Africa. They are used in the packhouses and, based on the results of the current study, can add value as they can also control brown rot through both curative and protective actions. Additionally, potassium phosphite is already registered in South Africa as a preharvest fungicide to manage *Phytophthora* spp. but can add value to the product as it can be used as a postharvest product as well. Future studies should look at fludioxonil and azoxystrobin as a combination for brown rot control as it was already successfully tested on *Penicillium* citrus pathogens in postharvest studies (Kanetis and Adaskaveg, 2007). It should also specifically determine if the protective activity of the three respective fungicides can last longer than 48 hrs which could add additional value to the cold chain.

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TABLES AND FIGURES

Table 1: Analysis of variance (ANOVA) of mean percentage brown rot observed on lemon, orange and mandarin fruit when treated curatively 6, 12, 24 and 48 hrs after inoculation with *Phytophthora nicotianae* zoospores.

Source	Lemon				Orange				Mandarin			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Fungicide x Time	12	46728.44	3894.04	<.0001	12	27428.67	2285.72	<.0001	12	21751.59	1812.63	<.0001
Error	27	1725.98	63.93		27	1980.61	73.36		27	3438.95	127.37	
Corrected total	39	48454.43			39	29409.29			39	25190.54		

Table 2: Analysis of variance (ANOVA) of mean percentage brown rot observed on lemon, orange and mandarin fruit when treated protectively 6, 12, 24 and 48 hrs prior to inoculation with *P. nicotianae* zoospores.

Source	Lemon				Orange				Mandarin			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Fungicide x Time	12	27743.49	2311.96	<.0001	12	38007.38	3167.28	<.0001	12	23358.22	1946.52	<.0001
Error	27	2929.69	108.51		27	4353.30	161.23		27	688.66	25.51	
Corrected total	39	30673.18			39	42360.68			39	24046.88		

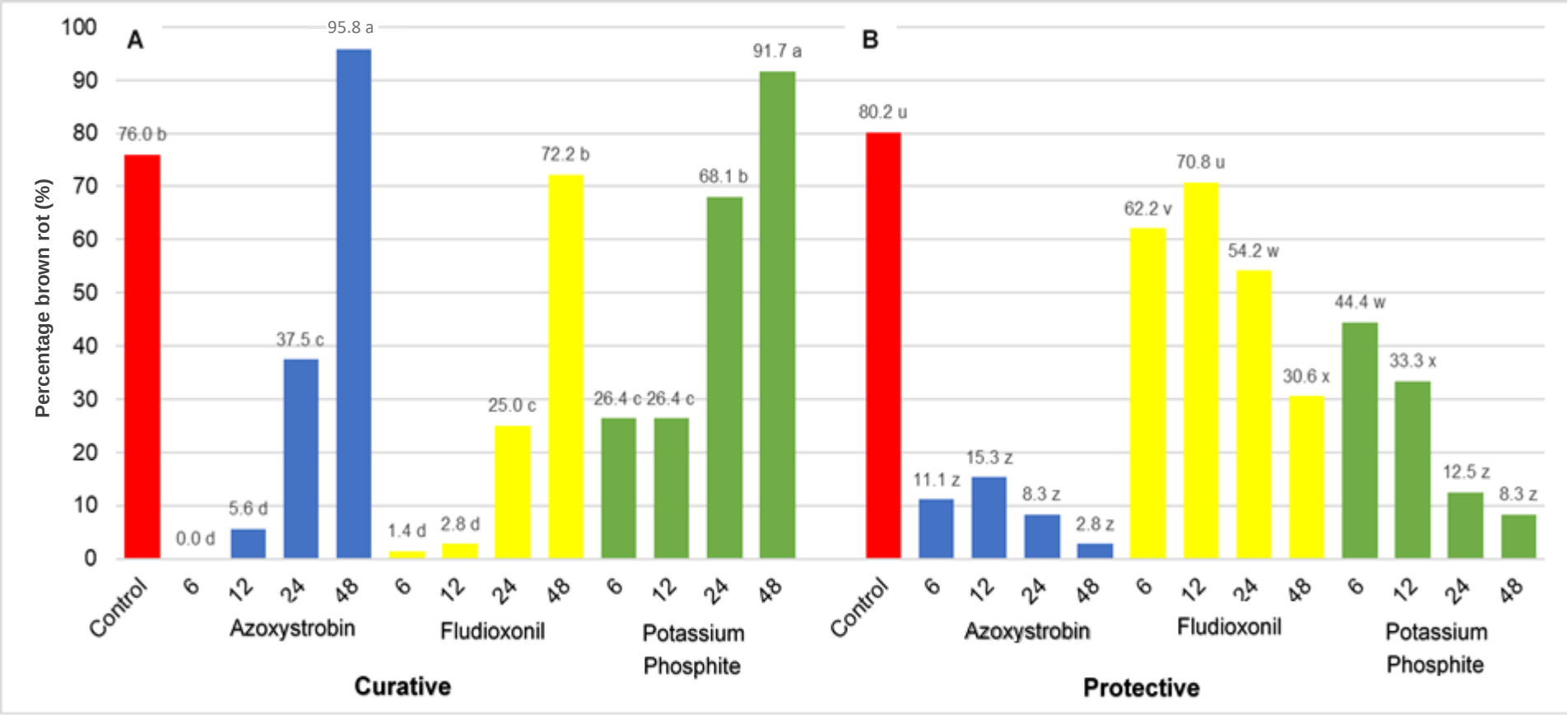

Figure 1: Mean percentage brown rot that developed on lemons, when fruit was treated curatively (A) with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.

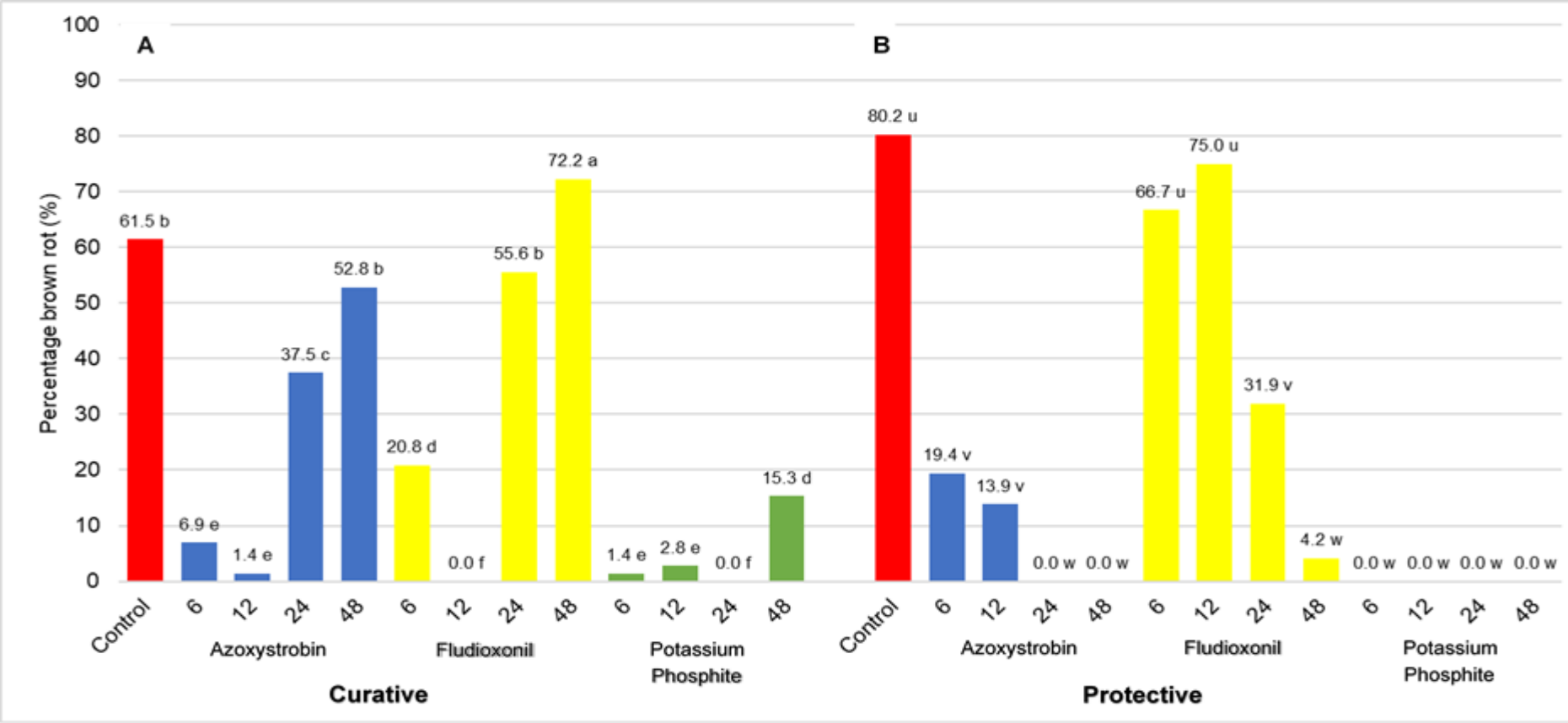

Figure 2. Mean percentage brown rot that developed on oranges when fruit was treated curatively (A)with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.

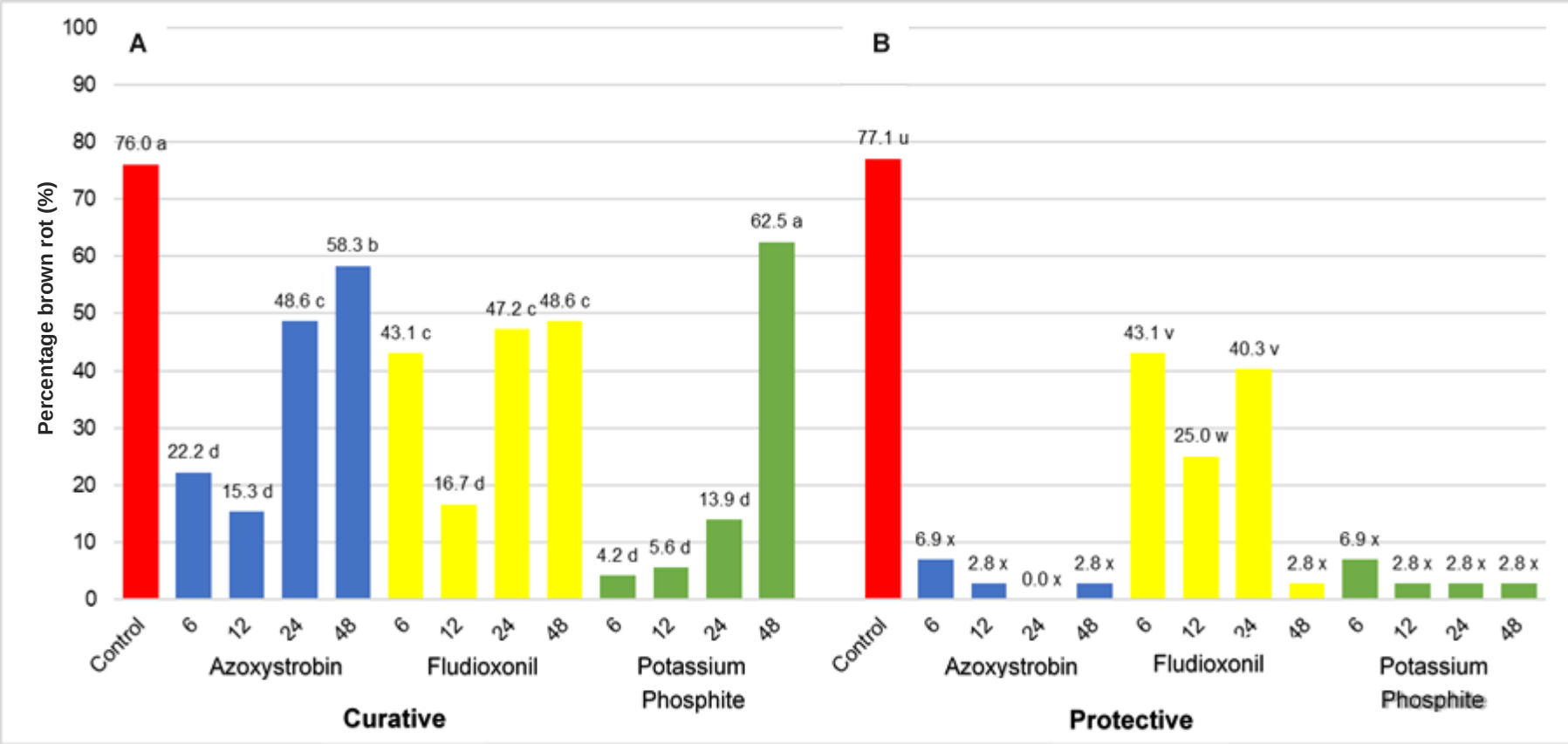

Figure 3. Mean percentage brown rot that developed on mandarins when fruit was treated curatively (A) with azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs after inoculation with *P. nicotianae* zoospores and protectively (B), when fruit was treated with either azoxystrobin, fludioxonil or potassium phosphite 6, 12, 24 or 48 hrs before inoculation with *P. nicotianae* zoospores.

Table 3: Analysis of variance (ANOVA) of mean percentage brown rot developing on lemon, orange and mandarin fruit treated with either azoxystrobin or fludioxonil amended wax prior to being exposed to brown rot symptomatic fruit while packed in a fruit carton.

Source	Lemon				Orange				Mandarin			
	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value	DF	Sum of Squares	Mean Square	P-value
Fungicide	2	3101.85	1550.93	0.0137	2	4178.24	2089.12	0.0018	2	81.019	40.51	0.5320
Error	9	1944.44	216.05		9	1354.17	150.46		9	538.19	59.80	
Corrected total	11	5046.30			11	5532.41			11	619.21		

Table 4: Mean percentage brown rot developing on lemons, oranges and mandarins treated with either azoxystrobin, fludioxonil or unamended wax before exposure to fruit with *P. nicotianae* infected fruit in a carton.

Wax treatment	N	Lemons	Oranges	Mandarins
Azoxystrobin	4	41.67 b	56.25 m	91.67 z
Fludioxonil	4	70.83 a	89.58 n	95.83 z
Control	4	79.17 a	100 n	97.92 z
LSD		23.51	19.62	12.37

[1]Means followed by the same letter are not significantly different.